3. Materials and Methods

Four major experiments were conducted in an attempt to develop a laboratory-scale growth model system for *Dicentrarchus labrax*, which would provide the essential framework conditions during axenical egg hatching and allow a successful performance of a gnotobiotically grown sea bass larvae.

Experiment 1 deals with the effects of glutaraldehyde egg surface disinfection, with further addition of rifampicin, ampicillin, kanamycin and tetracycline antibiotics to the culture medium, while experiments 2 and 3 are related to the effect of iodine disinfection.

In experiment 4, the procedure which secured the highest survival rate in the previous trials, was then applied to different treatment regimes, such as larvae being exposed to defined amounts of selected individual microorganism strains (*Aeromonas hydrophila* LVS 3 and *Saccharomyces cerevisiae*). Moreover, in an attempt to prevent sedimentation of the added strains, new rotating devices were tested.

In the first three experimental sets, the treatment groups were designed to evaluate the efficiency of different surface chemical treatments and to assess whether the effect of disinfection in combination with antibiotic supplements, would reduce the bacterial contaminants, thus improving early larvae survival.

The bacterial load on eggs and larvae was permanently evaluated, and, to ensure that antibiotics or antibacterial solutions did not affect the morphogenesis processes of the target organism in any circumstances, morphological analysis was eventually employed.

Once the first experimental sets were established, within the fourth experimental set, considerable efforts were made to evaluate if the introductory strain regimes and new rotary devices had considerable influence on the larvae performance and survival rates.

It should be emphasized that for each experiment, eggs from one single egg batch were used. The eggs were obtained from natural spawning of captive broodstock.

Unless otherwise stated, the natural seawater used in all experimental setups was previously filtered (0.2 µm), adjusted to a salinity of 32 $g.L^{-1}$, sterilized by autoclavation (120ºC for 20 minutes) and finally acclimatized to ambient temperature before use.

All procedures throughout the experiments were aseptically conducted. Therefore, all the material was previously sterilized by autoclaving (120ºC for 20 min), and all handling performed under axenic conditions, on a sterile bench (disinfected with 70% alcohol), within a laminar flow hood chamber.

Common General Procedures

3.1. SUPPLY OF *D.labrax* EGGS AND REARING CONDITIONS

The *D.labrax* eggs were supplied by the commercial hatchery, "Ecloserie Marine de Gravelines" (Dunkerque – France). Within some hours after collection, the eggs were transported in an isothermal container (cooler) to a temperature-controlled room in the Laboratory of Aquaculture & Artemia Reference Center (Ghent – Belgium).

Upon arrival, the eggs were acclimatized to a temperature of 15ºC, and transferred to a conical incubator tank with 5 L of filtered[7] UV-irradiated seawater (with a constant flow-through renewal extent rate of 100%, for a period of 1 – 2 hours). Throughout this period, water temperature was maintained at 15ºC ± 1ºC and a proper low aeration supplied through air-stones.

After acclimatization for more or less 5 hours, the egg density in the conical incubator was estimated. Egg assessment, was accomplished by counting the number of eggs present in 6 sub-samples of 10 ml each, and multiplying the average value by the total tank volume.

For the egg density inference to be statistically accurate, eggs should be homogeneously distributed within the water column. To ensure such distribution pattern, the eggs ought to be kept in suspension by a light aeration (Mbaluka, 2002).

3.2. CHEMICAL TREATMENTS ON THE *D.labrax* EGGS

After egg density assessment, the required volume was sieved (following manufacture's information: 1 dose 100 ppm antimicrobial solution, for 1200 eggs approximately), using a sterile 300 µm nylon mesh size, and the collected eggs rinsed with 32 $g.L^{-1}$ filtered autoclaved sea water (FASW), so as to remove the organic material, which may reduce the bactericidal activity of the disinfectants (Salvesen & Vadstein, 1995; Salvesen *et al.*, 1997; Elliot & Amend, 1978 *in* Grotmol *et al.*, 2003).

Afterwards, the eggs were divided into treatment groups (Table 3.1, with exception of the G+4A treatment group), and each group transferred to a designated disinfection solution. It should be emphasized that the control groups underwent identical physical handling as the disinfected groups.

During disinfection procedures (described below) the eggs were gently agitated every second minute, to ensure that their entire surface was exposed to the antimicrobial agent around them (Salvesen & Vadstein, 1995). All procedures were carried under dim light conditions, at 15-17ºC.

Subsequent to the surface disinfection treatment, the antimicrobial solutions were drained off and the eggs carefully washed in four successive baths (20 s each), of approximately 700 ml of FASW, to ensure the wash out of any remaining disinfectant residues from the eggs surface (Figure 3.1).

[7] Seawater was filtered using a Sartobran *P* unit, adapted with a 0.45 µm pre-filter and a 0.2 µm final filter (Model type: OO, Sartorius AG, Götingen – Germany), to remove most bacteria and particle organic nutrients.

Thereafter, each treated group was evenly split into autoclaved 500 ml glass bottles, each with six replicates containing approximately 600 eggs in 400 ml FASW (32 $g.L^{-1}$).

A low level of filtered air (0.2 µm membrane filter) was supplied through sterile tubes to all incubation bottles.

Throughout the following days, the treatment bottles were kept undisturbed (except when bottles were gently stirred once each day, so the eggs wouldn't agglomerate) and maintained in complete darkness[8] at 15ºC ± 1ºC, to allow hatching. The standard incubation temperature and air supply are of major importance to a successful egg development, it may determine certain morphological features as well as the hatching rate and also the behaviour and growth rates of the larvae upon hatching (Bagenal & Braum, 1970 *in* Hansen & Falk-Petersen, 2001).

3.2.1. Glutaraldehyde Disinfection Treatment

The rinsed eggs were submerged and treated for 5 minutes with a 200 $mg.L^{-1}$ glutaraldehyde solution (working concentration of 50% v/v) in 500 ml of FASW, using a sterile sieve (300 µm mesh size).

3.2.2. Glutaraldehyde Disinfection Treatment with Further Addition of Antibiotics

To some of the eggs previously disinfected with glutaraldehyde (100 $mg.L^{-1}$), 10 $mg.L^{-1}$ of ampicillin and 10 $mg.L^{-1}$ rifampicin were added (Appendix A).

3.2.3. Iodophor Disinfection Treatment

Following a similar protocol, for the iodine treatment group the rinsed eggs were immersed for 5 min in a Iodophor Romeïod solution of 6.25 $mg.L^{-1}$ active iodine (iodine working concentration 0.5% v/v). Afterwards, the iodine was neutralized by the addition of a sodium thiosulfate solution (1.5 $g.L^{-1}$), until a change to a clear coloration indicated neutralization.

Table 3.1. – Treatment group details from the experiments in the effect of different chemical treatments on the bacterial load of *D.labrax* eggs and larvae, and respective survival rates.

Group	Treatment group details
C	Control treatment, consisting of non-disinfected eggs;
G	Glutaraldehyde disinfection treatment, in which eggs were exposed to 100 $mg.L^{-1}$ glutaraldehyde solution for 5 min;
G+2A	Glutaraldehyde disinfection with 2 antibiotics treatment, where eggs previously disinfected with glutaraldehyde treatment received a supplement of 10 $mg.L^{-1}$ ampicillin and 10 $mg.L^{-1}$ rifampicin;
G+4A	Glutaraldehyde disinfection with 4 antibiotics treatment, consisting of previously disinfection with 100 $mg.L^{-1}$ glutaraldehyde solution, followed by the addition of 10 $mg.L^{-1}$ ampicillin, rifampicin, kanamycin and tetracycline each;
I	Iodine disinfection treatment, in which eggs were exposed to approximately 6 $mg.L^{-1}$ iodine solution for 5 min and afterwards neutralized with sodium thiosulfate solution.

[8] It is standard procedure to keep sea bass larvae in complete darkness, until complete yolk sac absorption occurs (Moretti *et al.*, 1999). Low intensity light is known to induce a delay in the onset of sea bass feeding behaviour (Cerqueira & Chatain, 1991; Cuvier-Péres *et al.*, 2000), enabling feeding postponement until the last practical moment (Jonhson & Katavic, 1986).

1st to 3rd Experimental Design Setups – AXENICALLY GROWN SEA BASS LARVAE

3.3. HATCHABILITY OF *D.labrax* EGGS AND LARVAE SURVIVAL AFTER DISINFECTION TREATMENTS

On the hatching day[9], sixty (6 x 10 larvae) newborn larvae from each treatment bottle were randomly chosen and gently transferred[10], one by one, to a 24-well tissue culture sterilized test plate (TPP, Trasadingen – Switzerland).

Hatched larvae from the G+2A disinfection treatment bottles were divided into two new multi-well treatment groups, namely: G+2A – Glutaraldehyde disinfection with further addition of ampicillin and rifampicin treatment & G+4A – Glutaraldehyde disinfection with posterior addition of ampicillin, rifampicin, kanamycin and tetracycline treatment (Table 3.1 and Figure 3.1). Ten milligrams per litter of each particular antibiotic was added to both treatment groups.

For each treatment group, five multi-well replicates and one additional multi-well plate with 50 larvae (5 x 10 larvae for later histomorphological sampling), were prepared.

The larval stocking density was 1 larva per 200 µl, and FASW was added to each well. To prevent evaporation of the medium and avoid contamination, the multi-well plates were covered with parafilm (Salvesen & Vadstein, 1995; Panini *et al.*, 2001).

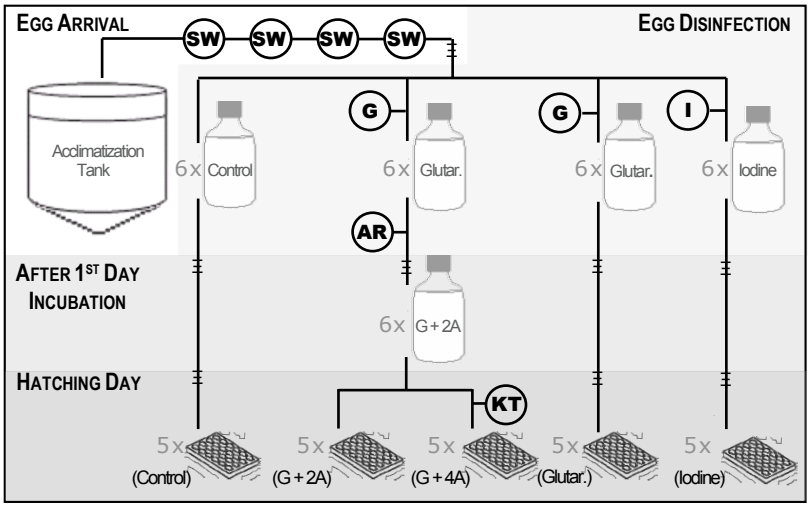

Figure 3.1. – Schematic diagram with the different chemical disinfection treatments, to which *D.labrax* eggs and larvae were subjected.

Legend: SW, rinsing procedures with 32 g.L^{-1} FASW; *G*, glutaraldehyde disinfection treatment; *G+2A* & *G+4A*, glutaraldehyde disinfection with 2 or 4 antibiotics treatment group; *I*, iodine disinfection treatment; *AR*, ampicillin and rifampicine antibiotic supplement; *KT*, kanamycin and tetracycline antibiotic supplement; Ξ, denotes a sampling instant. *Note:* Glutaraldehyde solely disinfection treatment, only occurred on the first experimental set, as the Iodine disinfection treatment only took place on the second and third sets.

The larvae were kept in complete darkness in a temperature-controlled (15ºC ± 1ºC) room, except during periods of larvae monitoring, when the room was illuminated by a dim red light (23V–15W).

Sudden outbreaks and other stress related factors, such as abrupt movements, by which larvae are mostly affected, were avoided.

[9] The day, by which, at least 50% of the eggs in the control treatment (C) had hatched.
[10] The micropipettes used for the transfer procedure were fitted with sterile cut-off pipette tips, to avoid damage to the larvae. Furthermore, to avoid cross-contamination among groups, a different sterile pipette tip was used for each replicate.

3.3.1. Hatching Success and Larvae Survival Rate

At the end of the egg incubation period, hatched larvae from all treatment bottles were counted. Day 0 was defined as the day by which at least 50% of the eggs in the control treatment (C) had hatched. The hatching percentage was calculated as the number of hatched larvae divided by the total number of incubated eggs. This ratio, integrates both the embryos mortality and the inability of living embryos to hatch (Salvesen & Vadstein, 1995).

Every second day, until the end of the experiment (DAH 11)[11], the larvae from 1 multi-well plate per treatment were monitored, and the survival rate of viable larvae determined. Observations were performed by means of a binocular magnifying glass, under aseptic conditions.

3.4. EFFECT OF DIFFERENT CHEMICAL TREATMENTS ON THE BACTERIAL LOAD OF *D.labrax* EGGS AND LARVAE

In order to evaluate the treatments' success and to verify the germ-free state of eggs and fish larvae throughout the growth cycle, a series of samples were taken for later microbiological analysis. The sampling programme with detailed information on the different disinfection treatment groups from which the samples were taken, is shown in Figure 3.2.

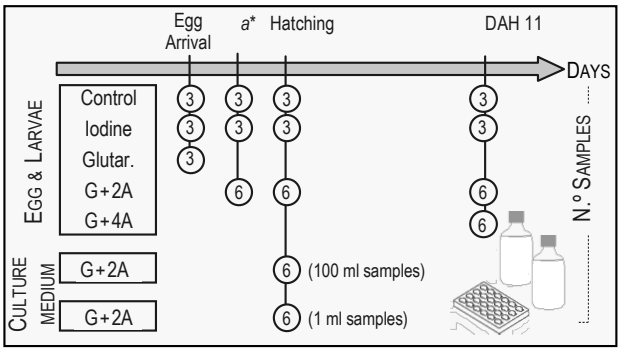

Figure 3.2. – Sampling schedule for microbiological analysis (adapted from Nicolas *et al.*, 1989), in respect to the first and second experimental sets.

*Legend: a**, after 24 hours egg incubation; *DAH*, days after hatching.

Absence of bacteria was monitored by the combination of two distinct assays based on similar principles, such as microbiological plating and turbidity tests.

3.4.1. Disinfection Efficiency Tests

In order to determine the presence of bacteria in the incubation culture medium, at the end of egg incubation, 1 ml samples were aseptically removed from each replicate bottle of the glutaraldehyde disinfection treatment with further addition of antibiotics (G+2A), and added to sterile capped tubes containing 9 ml 10% MB (Difco Laboratories™ - Marine Broth 2216) Appendix A.

Soon after, the tubes were set to incubate at 20, 25 and 28ºC, for succeeding intervals of 24 hours. Turbidity was evaluated at the end of each incubation period.

Likewise, subsequent to larvae stocking in multi-well plates, two 100 ml sub-samples from the culture medium of each replicate of the G+2A treatment bottles, were filtered on 0.2 µm sterile filters (using Büchner filters). The filters were then plated on 10% MA and 10% MA+R+A plates plates (10% Marine Agar supplemented with 10 mg.L^{-1} Rifampicin and 10 mg.L^{-1} Ampicillin antibiotics) Appendix A, and set to

[11] DAH – Days after hatching (e.g. DAH 7, seven days after hatching).

incubate according to an identical incubation schedule protocol as described above. Appraisal for Colony Forming Units (CFU) was done after 24, 48 and 72 hours incubation.

In order to get a more sensitive reading from the bacterial load to which the larvae were subjected, every second day after survival estimation, 1 ml of 30% MB was added to each well from the assessed multi-well plates. The respective plates were incubated at 20ºC for 24 hours, subsequently increased to 25ºC over 24 hours incubation and finally to 28ºC, for another 24 hours. Turbidity was evaluated at the end of each incubation period as response signs of bacterial growth.

3.4.2. Methods Used to Verify Axenic State

On the day the eggs were stoked, just after rinsing and after the disinfection procedures, 3 replicates of 30 eggs were removed from each treatment group (Figure 3.2). To avoid cross-contamination among groups, a different 25 ml sterile pipette was used for each replicate.

After one-day incubation, another 3 replicates were taken from each treatment group, with the exception of the glutaraldehyde disinfection treatments with further addition of 2 antibiotics, from which 6 replicates of 30 eggs were taken (Figure 3.2).

On the day of hatching and eleven days after hatching (DAH 11) a similar procedure was performed, but instead of the 30 eggs per replicate, 30 larvae were removed from each treatment group.

The samples were kept, for a short period, in sterile falcon tubes (TRP®, γ-irradiated). Soon after, each sample was aseptically sieved (using a 300 μm nylon mesh size) and subsequently rinsed with sterile Nine Salt Solution (NSS Appendix A), so as to remove non-adherent microorganisms from the eggs (Bergh & Jelmert, 1996; Mbaluka, 2002; Verner-Jeffreys *et al.*, 2006). Subsequently, samples were transferred to separate sterile stomacher plastic sample bags, containing 10 ml of NSS.

Thereafter, eggs and larvae samples were separately homogenised using a Seward Stomacher 400 Lab Blender Mixer (Type BA7021, Seaward Medical London - UK) at normal paddle speed for 120 seconds and at high speed for another 120 seconds. Each homogenised solution was serially tenfold diluted[12] with sterile NSS, and dilutions spread plated on 10% MA (Marine Agar Appendix A) by a Spiral Plater (Model DU, Spiral Systems Inc., Cincinnati, OH – USA). All homogenate dilutions were spread plated in duplicate to verify the reproducibility of the results.

From the day of hatching (DAH 0) onwards, dilutions of the glutaraldehyde disinfection treatments with further addition of antibiotics were spread plated onto 10% MA and on 10% MA+R+A.

Afterwards all plates were incubated for 24 hours at 20ºC. Plates were subsequently incubated at 25ºC over 24 hours of incubation, and finally at 28ºC for another 24 hours. Viable counts were expressed as CFU.ml^{-1} and counts above 30 CFU per plate were used for analysis[12].

[12] According to Gunasekaran (1995) after incubation, plates should contain between 30-300 cells.ml^{-1} so the colony forming units assessment would be accurate. Following such concept, it is advisable to make a direct microscopic count prior to spread plating, so that appropriate serial dilutions from the homogenates are prepared.

3.5. MORPHOLOGICAL FEATURES FROM *D.labrax* LARVAE, APPLIED TO THE STUDY ON THE EFFECT OF DIFFERENT CHEMICAL TREATMENTS

Since hatching day, a sample of 10 newborn larvae was daily taken from each multi-well treatment group (which was prepared for later histological sampling), and transferred to 2 ml sterile separate eppendorfs.

From the mentioned larvae samples, a small fraction from the control (C) and the glutaraldehyde disinfection with 2 antibiotic (G+2A) treatments, from the second experimental set, were considered for Scanning Electron Microscopy (SEM) observations.

3.5.1. Fixation Procedures

The samples were then centrifuged at 2000 rotations per minute (rpm), for 2 minutes in a Sanyo Harrier 16/80 Centrifuge (Model: MSBO80.CR2.K, Jepson Bolton & Co Ltd. – UK), the supernatant removed and the pellet resuspended in 2 ml of Phosphate-Buffered Saline (PBS Appendix A) solution. The same procedure was carried out another 3 times.

Subsequently, each sample was fixed in 2 ml of Neutral Buffered Formaldehyde solution Appendix A at room temperature, and then kept at 4ºC for at least 24–72 hours, prior to later histological assays conducted at the Faculty of Veterinary Medicine – Department of Morphology – Laboratory of Histology (Ghent University, Merelbeke – Belgium).

Once at the Merelbeke laboratory, after the fish larvae treatment groups had been selected for analysis, they were identified throughout all stages by a tag identification or label[13] bearing the number given to the specimen.

3.5.2. Histomorphological Procedures for Scanning Electron Microscopy

Larvae fixed in Neutral Buffered Formaldehyde solution, were further fixed with a modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde) in HEPES buffer Appendix A solution (pH 7.2), for 24–48 hours at 5ºC.

After rinsing the samples with distilled water, the larvae were post-fixed in 1% osmium tetroxide[14] (Osmium [VIII] oxide, #1.24505.0001 [1g], VWR International – Belgium) for another 2 hours, at room temperature. Later, the fixative solution was removed from the larvae samples and placed in an appropriate hazard waste container.

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13 Fish larvae samples were identified by means of numbers, upon arrival at the Morphology laboratory. The adoption of a well-organized system of sample labelling, ensures that there is little danger of incorrect reporting, due to errors or exchanges of sample identity (Gordon & Brandbury, 1977).

14 Osmium Tetroxide (OsO_4) is highly oxidizing and highly toxic (T^+ - EU classification). Extremely harmful by inhalation, when in contact with skin/eyes, can be lethal if ingested. When sensitization occurs, the effects are usually going to be irreversible. It is also considered as a possible mutagen (Material Safety Data Sheet – VWR International).

Subsequent to fixation, larvae samples were briefly rinsed with distillate water and carefully transferred to individual[15] microporous specimen capsules (Model Type: 30 µm, SPI Supplies Inc., West Chester – USA).

Following dehydration in graded alcohol, larvae samples were then set in the specimen pressure chamber of the Critical Point Dryer (Model: CPD 030, Balzers Union Aktiengesellschaft, Liechtenstein - Switzerland), to dry with liquid CO_2. To ensure good electrical conductivity for SEM analysis, the samples were afterwards coated with a thin film of platinum by sputter-coating (Model: JFC-1300 Autofine Coater, JEOL Ltd. – Japan).

Qualitative analysis was accomplished by means of a Scanning Electron Microscope (Model: JSM-5600 LV, JEOL Ltd. – Japan) provided with elemental analysis and image analysis systems (JEOL SEM User Interface, Version 1.31). Surface morphology images from the coated specimens were methodically captured by using an adapted digital camera.

3.5.3. Morphometric Measurements and Anatomical Observation

Morphometric parameters (Figure 3.3) follow those used by Barnabé (1976), Wood (2000), Koumoundouros *et al.* (2001), Alemany (2003) and Machinandiarena *et al.* (2003).

Morphometric characteristics (Table 3.2), from each representative specimen, were assessed in at least three micrographs. Larval measurements were performed to the nearest 0.1 µm, by using the analysis image processing software for the SEM microscope. All lengths were measured parallel to the longitudinal axis of the body, while depths were measured perpendicular to this axis. The yolk-sac volume was calculated from the formula of a prolate spheroid, $YsV = (\pi/6)\,YsL \cdot YsD^2$ (Blaxter, 1963 *in* Koumoundouros, 2001).

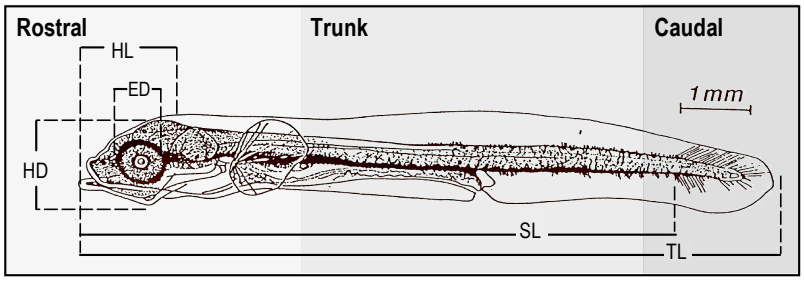

Figure 3.3. – Morphometric measurements and the 3 segments (Rostral, Trunk and Caudal) defined throughout the histomorphological analysis – Drawing from a *D.labrax* larva specimen at 15 DAH (adapted from Barnabé *et al*., 1976).

Legend: ED, eye diameter; *HD*, head depth; *HL*, head length; *SL*, standard length; *TL*, total length).

For each individual, nine morphometric characteristics were examined as whether present or not. These characteristics were: (a) pectoral fin buds, (b) the differentiation of the primordial margin fin-fold into the three single fins, (c) the anus aperture, (d) the definition of the optic capsule, (e) the fine delineation of the nostrils, (f) the opening of the mouth, (g) the onset of notochord flexion (pre-flexion vs. flexion stage), (h) the fully completed notochord flexion (flexion vs. post-flexion stage), (i) the

[15] Most reliable at this labelling stage, is the use of thin white cards with writing in soft pencil that withstands all the fluids used in the tissue processing (Gordon & Bradbury, 1977).

appearing of teeth and scales. The status of each one of these characteristics was noted for each micrograph taken.

Table 3.2. – Morphometric characteristics measured in the present study.

Character	Abbreviation	Description
Eye diameter	ED	Parallel to the longitudinal axis of the body;
Head depth	HD	The maximum depth of the head;
Head length	HL	From tip of the snout to the posterior margin of the cleithrum;
Pre-anal length	prAnl	From the tip of the snout to the anus (along midline of the body);
Standard length	SL	From tip of the snout to the concealed base of the caudal fin rays;
Total length	TL	From tip of the snout to the tip of the notochord in pre-flexion larvae, and to the end of the caudal fin in post flexion individuals (along the midline of the body);
Yolk-sac depth	YsD	The maximum depth of the yolk-sac;
Yolk-sac length	YsL	The maximum length of the yolk-sac;

4th Experimental Design Setup – EVALUATION OF DIFFERENT HOLDING REGIMES ON THE SURVIVAL RATE OF GNOTOBIOTICALLY GROWN SEA BASS LARVAE

3.6. *D.labrax* AXENIC LARVAE

After acclimatization, the eggs were split into 2 treatment groups, namely the Control (C) consisting of non-disinfected eggs, and the Glutaraldehyde disinfection with 2 antibiotics treatment (G+2A), where eggs previously disinfected with glutaraldehyde received a further supplement of 10 $mg.L^{-1}$ ampicillin and 10 $mg.L^{-1}$ rifampicin (following the protocol mentioned in Section 3.2). In all other respects, the control and the disinfected groups were treated in a manner similar to the previously employed in the first experimental setups.

Ninety-six eggs from each treatment group were chosen at random and gently transferred, one by one, to a 96-well tissue culture sterilized test plate (TPP, Trasadingen – Switzerland). A final stocking density of 1 egg per 150 µl FASW was kept.

To avoid external contamination and prevent evaporation of the incubation medium, the multi-well plates were covered with parafilm (Salvesen & Vadstein, 1995; Panini *et al.*, 2001).

Further, each treatment lot was evenly split into autoclaved 500 ml glass bottles, each containing approximately 600 eggs in 400 ml FASW, the control treatment group with 6 replicate bottles and the disinfected treatment group with 12 replicates (Figure 3.4).

Filtered air (0.2 µm membrane filter) was constantly supplied to all treatment bottles, through sterile tubes.

Until hatching, multi-well plates and incubation bottles from both treatment groups were kept undisturbed at 15ºC ± 1ºC, in complete darkness.

3.6.1. Hatching Success

Later on, the hatching percentage from each multi-well treatment plate was determined, based on the total number of stocked eggs and the number of assessed fry. A row of 12 wells was considered as one single replicate.

3.6.2. Methods Used to Verify Axenic State

In order to evaluate the treatment success, 24 hours after surface disinfection, 30 larvae from each bottle were sampled, homogenised and spiral plated on 10% MA, as described in Section 3.4.2.

Assessment for viable colony formation (CFU) was done after 72 hours incubation.

3.7. INFLUENCE OF ROTARY MOTION DEVICES ON *D.labrax* LARVAE PERFORMANCE

At hatching, 10 newly hatched larvae were randomly picked from the incubation bottles and gently shifted, one by one, into 10 ml polystyrene sterilised vials (by overnight exposure to UV light) with white screw caps. Each treatment had four replicates of 4 vials, each one containing 10 ml FASW.

Subsequently, the polystyrene vials were capped and placed on two different rotation devices. Sixteen vials from each treatment group, were placed on Rotor A, turning at 1 rpm in a perpendicular direction to the longitudinal axis of the vials. Another 16 vials were placed on a second rotor (Rotor B), turning at 4 rpm in a parallel direction to the longitudinal axis of the vials (Figure 3.4).

On the same day, four 24-well sterilized test plates, each with fifty larvae (5 wells x 10 larvae), were prepared for both treatment groups. A stocking density of 10 larvae per 150 µl FASW was attained.

Plates were then covered with parafilm, and left undisturbed until later larvae monitoring. Sudden outbreaks and other stress related factors such as abrupt shifting movements, by which larvae are mostly affected, were avoided.

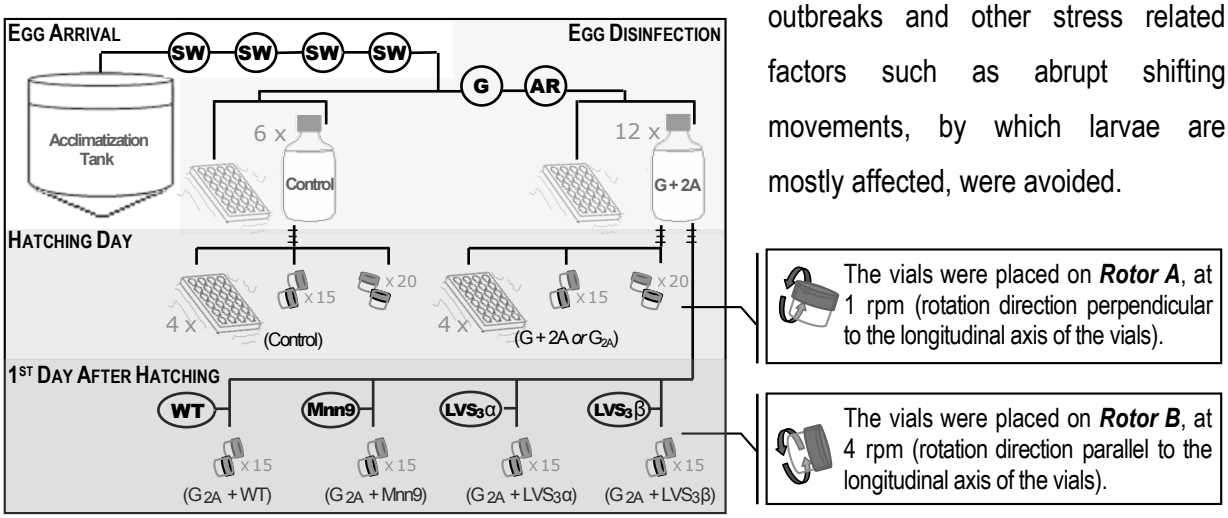

Figure 3.4. – Schematic diagram from the different treatment holding regimes to which *D.labrax* eggs and larvae were submitted.

Legend: SW, rinsing procedures with 32 g.L^{-1} FASW; *G*, glutaraldehyde disinfection treatment; *AR*, ampicillin and rifampicine antibiotic supplement; *G+2A or G$_{2A}$*, glutaraldehyde disinfection with 2 antibiotics treatment group; *WT*, axenic wild type baker's yeast (*Saccharomyces cerevisiae*) strain added separatelly at 10^6 cells.ml^{-1}, *Mnn9*, axenic *S.cerevisiae* baker's yeast mnn9 isogenic mutant, added at 10^6 cells.ml^{-1}; *LVS$_3$ α*, addition of *Aeromonas hydrophila* LVS 3 (rifampicin resistant) axenic bacterial culture strain, obtaining a final concentration of approximately 10^4 CFU.ml^{-1}; *LVS$_3$ β*, addition of *A.hydrophila* LVS 3 (rifampicin resistant) axenic bacterial culture strain, obtaining a final concentration of approximately 10^5 CFU.ml^{-1}; ≡, denotes sampling instant.

The larvae from both static and dynamic setups were kept in a temperature-controlled (15ºC ± 1ºC) room, in complete obscurity. Over larvae monitoring and assessment periods, the room was exceptionally illuminated with a dim red light (23V–15W).

3.7.1. Larvae Survival Rate

Every second day until the end of the experiment (DAH 13), a replicate of 4 vials was carefully removed from each treatment group, and the survival rate for each rotating device determined.

In order to compare the outcome effect that rotary motion had on the larvae survival rate, with the one obtained from the motionless setup, larvae from 1 multi-well plate were monitored over those same days (DAH 3, 6, 9 and 13) and the number of living larvae recorded for both treatment groups.

3.7.2. Efficiency Trial Used to Confirm Axenic Status

To get a more sensitive reading from the axenic conditions to which the larvae were exposed, 1 ml of 30% MB was added, every second day after survival estimation, to each well from the assessed multi-well plates and to the polystyrene vials, regarding the G+2A treatment groups. Subsequently, they were set to incubate (following similar procedure as described in section 3.4.1).

Turbidity, being a sign of bacterial growth, was evaluated at the end of each incubation period.

3.8. EFFECT OF DIFFERENT MICROBIAL STRAINS ON GNOTOBIOTICALLY GROWN *D.labrax* LARVAE

3.8.1. Axenic Yeast Cultures

Two axenic baker's yeast (*Saccharomyces cerevisiae*) strains were evaluated on gnotobiotically grown sea bass larvae. A wild type strain (WT) [BY4741; genotype *Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*] and its mnn9 isogonic's mutant (Mnn9) [BY4741; genotype *Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* YPL050c::kanMX4] (Marques *et al.*, 2004a), both provided by the European *Saccharomyces cerevisiae* Archive or Functional Analysis – EUROSCARF (University of Frankfurt – Germany).

Both yeast strains were grown at 28ºC, on a complete Yeast Extract-Peptone-Dextrose (YEPD Appendix A) medium and maintained on agar slopes at 4ºC.

In order to check their potential viability[16], one week before the experiment took place, samples from both strains were spread in duplicate on YEPD agar plates. Inactivation was confirmed by growth absence after 5 days incubation at 28ºC.

Starting from a single colony, yeast cultures were grown in separate sterile Erlenmeyer's flasks and placed to incubate for 72 hours at 28ºC, on a constant gyratory shaker (Model G76, New Brunswick

[16] Earlier on, in order to determine the time-period strains normally needed to form macroscopically visible colonies, small scale tests were performed.

Scientific, New Jersey – USA) operating at 150 rpm. The WT strain was cultured in 20 ml YEPD broth, and the Mnn9 mutant in 20 ml Yeast Nitrogen Based broth medium (YNB Appendix A).

Three days after inoculation, within the early stationary growth phase[17], cell suspensions were harvested by centrifugation (5000 rpm for 10 min) and twice resuspended in 20 ml of FASW. Cell densities of such concentrate stock suspensions were determined by twice measuring the cell concentration, using a Bürker haemocytometer [(cells counted x number of squares counted x dilution factor) / volume measured by the 25 squares of the haemocytometer].

3.8.2. Bacteria Strains and Growth Conditions

Pure cultures from *Aeromonas hydrophila* – LVS 3 (Rifampicine and Ampicillin Resistant), were provided by the Laboratory of Microbial Ecology and Technology – LabMET (Ghent University – Belgium).

To assess their potential ability to grow, two weeks prior to the experimental setup, one loop-full of such suspension was streaked on marine agar supplemented with rifampicin and ampicillin antibiotics (MA+R+A), and incubated overnight at 28ºC.

Actively on-growing cultures were prepared by simply inoculating a single colony from those slant plates, into 20 ml fresh MB in sterile Erlenmeyer's flasks, and incubating at 28ºC under constant agitation (150 rpm).

After 24 hours incubation, bacteria suspensions were harvested by 15 min centrifugation at 1500 rpm, the supernatant discarded and the pellet rinsed and twice resuspended in 20 ml FASW.

Assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells.ml^{-1} (MacFahrland standard *in* Verschuere *et al.*, 1999), the densities of the new bacterial suspensions were estimated by absorbency measurements at 550 nm, with a Spectronic GENESYS 20 Vis spectrophotometer (Thermo Electron Co. – USA).

3.8.3. Inoculation of the Selected Strains

On the day after hatching, 10 larvae from the remaining G+2A incubation bottles were transferred to new sterile vials, each containing 10 ml of FASW.

Yeast and bacteria cultures were previously cultivated, as described in Sections 3.8.1 and 3.8.2.

Immediately after the larvae transfer, each microbial suspension was separately inoculated into 16 vials, thus obtaining final cell densities as listed below (Table 3.3). In addition, one group consisting of 20 non-inoculated vials was used as control.

The flasks were capped and placed in a temperature-controlled room, on a rotation device at 4 rpm, preventing sedimentation of the individual microorganisms strains (Figure 3.4).

Larvae were exposed to constant darkness and a temperature of 15ºC ± 1ºC.

[17] The growth curve of each strain was established by regularly measuring their absorbance at 600 nm, in preceding experimental trials.

Table 3.3. – Treatment group details from the experiment on the effect of different individual microorganism strains supplied to gnotobiotically grown *D.labrax* larvae.

Group	Treatment group details*
WT	Wild type treatment, where gnotobiotic larvae were exposed to axenic wild type baker's yeast (*Sccharomyces. cerevisiae*) at 10^6 cells.ml^{-1};
Mnn 9	*S. cerevisiae* axenic *mnn9* isogenic mutant treatment, in which the gnotobiotic larva received a supplement of mnn9 at 10^6 cells.ml^{-1};
LVS$_3$ α	*Aeromonas hydrophila* LVS 3 (rifampicin resistant) α treatment, consisting of gnotobiotic larvae culture medium being inoculated with a LVS 3 bacterial strain, thus obtaining a final concentration of approximately 10^4 cells.ml^{-1};
LVS$_3$ β	*A.hydrophila* LVS 3 (rifampicin resistant) β treatment, wherein gnotobiotically grown larvae were exposed to approximately 10^5 cells.ml^{-1} of LVS 3 bacterial suspension.

*When treatment groups are being established, it is important to bear in mind that all diverged from previous G+2A treatment replicates (see Table 3.1 and Section 3.8.3 for further details).

Experiments Data Analysis

3.9. STATISTICAL ANALYSIS

Parametric assumptions were evaluated using Levene's test for homogeneity of variances and Shapiro-Wilk's for normality. After such assumptions were satisfied, differences in the CFU viable counts, hatching and survival rates of larvae amongst the different treatment groups, were assessed. Data were methodically compared by analysis of variance (ANOVA), following Tukey's multiple range tests, whenever significant differences were found, while when one or both tests checking normality and variance homogeneity failed, Kruskal-Wallis significant ranks were applied (followed by Mann-Whitney test for individual significant differences).

When it seemed pertinent, values from larval survival were arcsin-transformed [score+0.0001] (adapted from Fowler & Cohen, 1990), so that normal distribution and homocedasticity requirements were satisfied.

Statistical analysis was performed using SPSS[18] statistic software for Mac® OS X (Version 15.0, SPSS Inc., Chicago – USA), at 95% level of significance. The resulting figures were expressed as the mean ± standard deviation (SD).

[18] SPSS - Statistical Package for the Social Sciences.