LE VAN BAO DUY

Live food and use of probiotics in rabbit fish (*Siganus guttatus* Bloch, 1787) larviculture

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Applied Biological Sciences (Ph.D.)

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Duy Le.

SUMMARY

The rabbit fish (*S. guttatus*) can be found along the coastal area in Central Vietnam, especially at Thua Thien Hue, Quang Nam and Binh Dinh provinces. In aquaculture, rabbit fish is usually polycultured with the tiger shrimp (*Penaeus monodon*) and they use the residues from shrimp farming for food. Rabbit fish could also improve the water quality and reduce diseases of the tiger shrimp, without using chemicals and antibiotics. At the moment, aquaculture is still totally dependent on the wild collection of rabbit fish fingerlings, which does not meet the demand of fingerlings for aquaculture. In the past, the Vietnamese Aquaculture Research Institute I, Hue University, Nha Trang University and Can Tho University have carried out research on the artificial reproduction of rabbit fish. Unfortunately, there is no report yet on successful artificial reproduction. Therefore, with the huge demand for rabbit fish fingerlings for aquaculture, the study on the artificial reproduction is important for the development of rabbit fish culture in Vietnam. Hence, the main objective of this study is to close the life cycle of rabbit fish in captivity allowing for the production of fingerlings at commercial level.

In view of the very small mouth opening (80 µm) upon hatching, it is anticipated that rabbit fish larvae would need very small live prey. *Proales similis* is a live food candidate with the body size of 83 ± 11 µm in length and 40 ± 6 µm in width. Large-scale production of rabbit fish larvae would require a considerable amount of *P. similis* individuals. It is hypothesised that *P. similis* would heavily rely on a diet of bacteria and hence specific protocols would need to be developed for *P. similis*. The results showed that in the presence of the live probiotic mixture, both rotifer *P. similis* and *Brachionus rotundiformis* showed a better growth performance relative to the treatments without the probiotic mixture or those with the antibiotics. In addition, the growth performance of the very small rotifer *P. similis* is more dependent on proliferating bacterial community than the bigger rotifer *B. rotundiformis* in both experimental and large-scale culture conditions. The supplementation of these probiotic bacteria not only increased the production of the rotifers, but also had a regulating effect on the microbial community (MC).

After a specific large-scale culture protocol of *P. similis* was established, a series of experiments to optimize the rabbit fish larval rearing were conducted. The successful production of rabbit fish fingerlings is anticipated to require a specific live food feeding protocol. It is hypothesized that *P. similis* will be a better starter food for rabbit fish larvae than *B. rotundiformis.* After that, specific live food and probiotic feeding protocols with *P. similis* and *B. rotundiformis* from hatching to 240 hph followed by specific feeding protocol with *B. plicatilis* and *Artemia* from day 10 to 25 were tested. The data showed that *P. similis* was by far the better starter food for rabbit fish larvae than the *B. rotundiformis.* The feeding incidence was earlier and significantly higher survival was obtained in the treatment fed *P. similis,* than those fed *B. rotundiformis.* It also showed the importance of the *B. rotundiformis* on the survival of rabbit fish larvae when introduced into the culture system from day 6, compared to the survival of larvae fed *B. rotundiformis* at other time points. Furthermore, the feeding of probiotic-enriched rotifers to the larvae increased the larval survival compared to those fed non-enriched rotifers. Finally, the rearing protocols from day 10 to 25 is also reported. The results showed that rotifers still play an important role as food to the fish larvae beyond day 10. After that, *Artemia* was the most suitable food for the larvae. The feeding schemes including *Artemia* resulted in significantly higher larval survival compared to those without *Artemia.* Compound diet could be fed to the larvae when combined with rotifers and *Artemia* for cost-effectiveness.

The successful closure of the life cycle of rabbit fish will allow performing further experiments on the mass production of fingerlings for aquaculture in Central Vietnam. It is hypothesized that the body LC-PUFAs composition and gut MC of wild larvae entering into lagoons and hatchery larvae will be different, and information on these differences might provide clues to further optimise rabbit fish larviculture. For that, the fatty acid composition of body tissue and the gut MC composition of hatchery and wild larvae of rabbit fish at 3 different locations over a period of 3 years were investigated. Results from this study showed that rabbit fish larvae contained high proportion of ARA content. The ARA proportion of wild larvae was stable between sampling locations over the three-year period, and no significant differences were detected in the ARA level between wild and hatchery samples. The DHA/EPA ratios in fish larvae were very high. The ratios varied between locations and years. Difference between temperatures at 3 locations was negatively correlated with the DHA content and

DHA/EPA ratio, but not EPA content. The results of Illumina analysis of wild samples showed that location affected the gut MC composition. The bacteria that were identified in the rabbit fish gut content were mainly belonging in the phylum Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria and Firmicutes. In addition, the gut MC composition of hatchery larvae, which were fed probiotic-enriched rotifers, was completely different from those in the gut of wild larvae. Next, relative genetic distances between the sampling locations ThuanAn and BinhDinh/QuangNam were bigger than those between BinhDinh-QuangNam. More importantly, the environmental temperature was correlated with larval gut MC, namely, differences between temperatures at 3 locations were positively correlated with genetic distances between MCs, suggesting that there might be a causative relationship. Food quality, water currents and temperature probably shape the larval gut MC in the wild. In the hatchery, it is shaped through the continuous supply of probiotics via feeding.
Despite the differences in the body DHA/EPA ratio and gut MC composition of gut MC of rabbit fish larvae between wild and the hatchery, the DHA/EPA ratio profile of hatchery larvae is still within the natural range and hatchery larvae are apparently growing well. Rabbit fish can harbor totally different gut MC and be apparently healthy (wild versus hatchery). The current larval rearing protocol is considered to constitute a solid basis for further optimalisation.

In summary, this is the first report of a specific protocol for the large-scale culture of *P. similis.* For the first time, the life cycle of rabbit fish in captivity was closed, allowing for the production of 15,000 – 20,000 fingerlings up to now. It is anticipated that the current protocol will allow for the development of a protocol for the production of fingerlings at commercial scale. In addition, the data generated from the PUFA content and gut MC composition of wild fingerlings entering into lagoons might provide clues for further research on nutritional requirements and MC management strategies of the rabbit fish larvae.

SAMENVATTING

De konijnvis (*S. guttatus*) kan worden aangetroffen langs de kust in Centraal Vietnam, meer bepaald in de Thua Thien Hue, Quang Nam en Binh Dinh provincies. Konijnvis wordt meestal gekweekt in een geïntegreerd kweeksysteem samen met de tijgergarnaal (*Penaeus monodon*) in de aquacultuur waarbij ze de restanten van de garnaalkweek gebruiken als voeder. De konijnvissen kunnen mogelijks ook de waterkwaliteit verbeteren en reduceren ziektes van de tijgergarnaal zonder gebruik te maken van chemicaliën en antibiotica. Momenteel is aquacultuur nog steeds compleet afhankelijk van konijnvisjuvenielen die uit de natuur worden gevangen wat echter de vraag voor juvenielen voor de aquacultuur niet dekt. In het verleden, werd onderzoek verricht naar de artificiële reproductie van konijnvis aan het Vietnamese Aquacultuur Onderzoeksinstituut I, de Hue Universiteit, de Nha Trang Universiteit en aan de Can Tho Universiteit. Jammer genoeg is er nog geen rapport over succesvolle artificiële reproductie. Omwille van de grote vraag naar konijnvisjuvenielen vanuit de aquacultuur, is de studie van de artificiële reproductie belangrijk voor de ontwikkeling van de konijnviskweek in Vietnam. Daarom is het hoofdobjectief van deze studie het sluiten van de levenscyclus van konijnvis in gevangenschap waardoor de productie van juvenielen op commercieel niveau mogelijk wordt.

Gezien de heel kleine mondopening (80 µm) na het ontluiken, wordt verwacht dat de konijnvislarven zeer kleine levende prooien zouden nodig hebben. *P. similis* is een kandidaat levend voer met een lichaamsgrootte van 83 ± 11 µm lang en 40 ± 6 µm breed. Grootschalige productie van konijnvislarven zou een aanzienlijke hoeveelheid *P. similis* individuen vereisen. Het wordt verondersteld dat *P. similis* sterk afhankelijk zou zijn van bacteriën als voedsel en dat daardoor specifieke protocollen zouden moeten ontwikkeld worden. De resultaten toonden aan dat in aanwezigheid van een levend probiotisch mengsel, beide rotifeersoorten een betere groeiprestatie hadden dan zonder levend probiotisch mengsel of wanneer antibiotica werden toegevoegd. Daarenboven is de groeiprestatie van de rotifeer *P. similis* meer afhankelijk van een groeiende bacteriële gemeenschap dan de rotifeer *B. rotundiformis* in zowel experimentele als massacultuur condities. De toediening van deze probiotische

bacteriën verhoogde niet alleen de rotifeerproductie, maar had eveneens een regulerend effect op de microbiota.

Nadat een specifiek protocol voor massakweek van *P. similis* werd bekomen, werden een reeks experimenten om de konijnvislarvenkweek te optimaliseren uitgevoerd. Het is te verwachten dat de succesvolle productie van konijnvisjuvenielen een specifiek levend voervoederprotocol zal nodig hebben. Er wordt verondersteld dat *P. similis* een beter startvoeder zal zijn voor konijnvislarven dan *B. rotundiformis.* Daarna werden aangepaste levend voer- en probiontenvoederprotocols met *P. similis* en *B. rotundiformis* vanaf de ontluiking tot dag 10, gevolgd door een soortspecifiek voederprotocol met *B. plicatilis* en *Artemia* van dag 10 tot dag 25 getest. Uit de bekomen data bleek dat *P. similis* een veel beter startvoeder was voor konijnvislarven dan het SS-type *B. rotundiformis.* De larven aten eerder en de overleving was significant hoger in de behandelingen waar *P. similis* werd gevoederd*,* dan in deze gevoederd met *B. rotundiformis.* Het toonde ook het belang het SS-type *B. rotundiformis* op de overleving en de totale lengte van de konijnvislarven aan wanneer deze werd geïntroduceerd in het kweeksysteem vanaf dag 6, wat resulteerde in de beste overleving, in vergelijking met de overleving van larven gevoederd met *B. rotundiformis* op andere momenten. Daarenboven, het voederen van probiont-aangerijkte rotiferen aan de larven verhoogde de overleving van de larven in vergelijking met deze die niet-probiont-aangerijkte rotiferen kregen. Finaal, de kweekprotocollen van dag 10 tot 25 werden ook gerapporteerd. De resultaten toonden aan dat rotiferen nog steeds een belangrijke rol vervullen als voeder voor de vislarven na dag 10. Daarna was *Artemia* het meest geschikte voeder van de geteste voeders voor de larven. De voederschema's met *Artemia* toonden eeu aan zienlijk hogere larvale overleving aan han deze zonder *Artemia*. Samengesteld, industrieel, voeder kon vervoederd worden aan de larven indien het gecombineerd werd met rotiferen en *Artemia* omwille van de kost efficiëntie.

Het succesvol sluiten van de levenscyclus van konijnvis liet verdere experimenten over massaproductie van pootvis voor aquacultuur in Vietnam toe. Het werd vooropgesteld dat de gegevens over de lange keten onverzadigde vetzuren

samenstelling van het lichaam en de samenstelling van de microbiële gemeenschap (MG) van het spijsverteringsstelsel van de juvenielen die de lagunes binnenzwemmen en van broedhuislarven verschillend zal zijn en dat informatie over deze verschillen kunnen aanwijzingen opleveren om de larvicultuur van konijnvis verder te optimaliseren. Daartoe werden de vetzuurcompositie van het lichaamsweefsel en de MG van het spijsverteringsstelsel van kwekerij-gekweekte en wilde larven van konijnvis uit drie verschillende locaties over een periode van drie jaar onderzocht. Resultaten van deze studie toonden aan dat konijnvislarven grote hoeveelheden ARA bevatten. De hoeveelheid ARA van de wilde larven was stabiel tussen de verschillende staalnamelocaties gedurende de drie jaar en er werden geen significante verschillen gevonden in het ARA niveau tussen stalen uit het wild en uit de kwekerij. De DHA/EPA verhoudingen in de vislarven waren heel hoog. De verhoudingen waren verschillend naargelang de locatie en het jaar van staalname. Het verschil in temperatuur op de drie locaties was negatief gecorreleerd met de DHA inhoud en DHA/EPA verhouding, maar niet met de EPA inhoud. De resultaten van de Illumina analyse van de stalen van wilde vissen toonden aan dat de locatie een effect had op de diversiteit en samenstelling van de spijsverteringsMG. De bacteriën die geïdentificeerd werden in de inhoud van het konijnvisspijsverteringsstelsel behoorden voornamelijk tot de Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria en Firmicutes fyla. Darenboven was de diversiteit en samenstelling van de spijsverteringsMG van kwekerij-gekweekte larven, die probiont-aangerijkte rotiferen werden gevoederd, compleet verschillend van deze in het spijsverteringsstelsel van de wilde larven. Daarnaast, de relatieve genetische afstanden tussen de staalnameplaatsen ThuanAn en BinhDinh/QuangNam waren groter dan deze tussen BinhDinh en QuangNam. Echter belangrijker; de omgevingstemperatuur was gecorreleerd met de spijsveteringsMG van de larven, namelijk, de verschillen tussen de temperatuur op de drie locaties waren positief gecorreleerd met de genetische afstand tussen MGs, wat eventueel duidt op een oorzakelijk verband. In het wild, voedselkwaliteit, waterstromingen en temperatuur modeleren waarschijnlijk de spijsverteringsMG. In de kwekerij, wordt deze gevormd door een continue toediening van probiotica via het voeder.

Ondanks de verschillen in de lichaams DHA/EPA-verhouding en de spijsveteringsMG samenstelling van konijnvislarven uit het wild of de kwekerij, is de DHA/EPAverhouding en profiel van larven uit de kwekerij nog steeds binnen de natuurlijke spreiding en groeien kwekerijlarven blijkbaar goed. Konijnvis kan een totaal verschillende spijsverteringsMG hebben en blijkbaar toch gezond zijn (natuurlijk versus kwekerij). Het huidige kweek protocol voor larven wordt gezien als een goede basis voor verdere optimalisatie. Samenvattend; dit is het eerste rapport over een specifiek protocol voor de massaproductie van *P. similis.* Voor het eerst werd de levenscyclus van konijnvis in gevangenschap gesloten, wat de productie van tot nu toe 15.000-20.000 juvenielen toeliet. Er wordt verwacht dat het huidige protocol zal toelaten een protocol voor de productie van juvenielen op commerciële schaal te ontwikkelen. Daarenboven, de gegevens over de lichaamsFAME en MG samenstelling van wilde juvenielen die naar de lagunes zwemmen zou eventueel aanwijzingen voor verdere onderzoeken over de nutritionele vereisten van en microbiële gemeenschap strategiën voor konijnvislarven kunnen verstrekken.

%	percentage
°C	degree Celsius
AB	antibiotic
AM, PM	ante meridiem, post meridiem
ANOVA	analysis of variance
ARA	arachidonic acid
BC	Bray-Curtis
BLAST	basic local alignment search tool
bp	base pairs
C, N	carbon, nitrogen
C.P.	Charoen Pokphand
CFU	colony forming unit
cm	centimeter
DARRD-TTH	Department of Agriculture and Rural Development – Thua Thien Hue
DGGE	denaturating gradient gel electrophoresis
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dph	day post hatching
DW	dryweight
Elovl	fatty acid elongase
EPA	eicosapentaenoic acid
et. al.	and others
Fad	fatty acid desaturase
FAME	fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nation
g	gram
g	relative centrifugal force for G force
GI	gastrointestinal

GmBH	gesellschaft mit beschrankter haftung
GSI	gonadosomatic index
h	hour
ha	hecta
HCG	human chorionic gonadotropin
hpf	hours post fertilization
hph	hours post hatching
HUFA	highly unsaturated fatty acids
ind	individual
kDa	kiloDalton
kg	kilogram
L	liter
L:D	light:dark
LAB	lactic acid bacteria
Lab.	laboratory
LH-RHa	luteinizing hormone-releasing hormone agonists
m	meter
m^3	cubic meter
MA	marine agar
MARD	Vietnamese Ministry of Agriculture and Rural Developmet
MC	microbial community
mg	milligram
min	minute
mL	milliliter
mm	millimeters
MRS	de Man, Rogosa and Sharpe
N/A	dada not available
NCBI	national center for biotechnology information

NMDS	non metric multidimensional scaling
OD	optical density
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PERMANOVA	permutational analysis of variance
Pg	picogram
pH	measure of the acidity of solution
PNR	point of no return
PUFA	poly unsaturated fatty acids
rho	rank correlation coefficient
rmANOVA	repeated measures analysis of variance
rRNA	ribosome ribonucleic acid
s	second
S.D.	standard deviation
SC	skewness coefficient
SPSS	statstistic package for the social sciences
STATA	general-purpose statistical software package
SS, S, L	supersmall, small, large
T-RFLP	terminal restriction fragment length polymorphism
TCBS	thiosulfate-citrate-bile salts-sucrose agar
TGGE	temperature gradient gel electrophoresis
TL	total length
Uni.	university
µg	microgram
µL	microliter
µm	micrometer

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES

CHAPTER 1

Introduction

1.1. Aquaculture – the current status

Aquaculture plays an important role in food supply, nutrition, income, livelihood, rural development and poverty alleviation for several hundred millions of people worldwide. In 2014, the fish consumption hit a record high of 20.1 kg per capita, which was a 10.2 kg increase compared to 1960s, and aquaculture accounted for half of the fish supply for human consumption. In the future, aquaculture is expected to supply food for nearly 10 billion people by 2050. Over the last 22 years, aquaculture production increased rapidly from 13.1 million tons in 1990 to 73.8 million tons in 2014 (Table 1.1) (FAO, 2016). The statistical data of 2014 mentioned that over 67% of the production was finfish (49.9 million tons), in which 43.6 million tons came from inland aquaculture and 6.3 million tons from mariculture. It was followed by the production of mollusks (21.8%, 16.1 million tons) and crustaceans (9.4%, 6.9 million tons) (Table 1.2) (Figure 1.1). By contrast, the fisheries production worldwide increased only slightly over the last decade, to 93.4 million tons in 2014 (FAO, 2016). In fact, most of the main fishing grounds around the world, such as China, Indonesia, Russia, USA, Japan, Peru and India have reached their maximum potential for capture fisheries. As a consequence, fisheries production will not be able to satisfy the human demand for aquatic products, and aquaculture production is anticipated to increase to 80 million tons by 2050 to meet global consumption (FAO, 2016).

Although aquaculture creates a lot of significant contributions to fish production for human consumption, still some critical issues need to be tackled, especially to foster sustainable growth of aquaculture. Firstly, intensive aquaculture goes together with water pollution and disease outbreaks. This might cause a decline in aquaculture production in some regions in the near future. Secondly, the rapid increase of aquaculture was correlated with a shortage of fish meal and fish oil for the aqua food industry. The use of one species for feeding another species is considered to be unsustainable. Lastly, the prophylactic use of chemicals and antibiotics in the water, *e.g.* disease treatments, not only creates long-term consequences directly to aquaculture species itself, but also to the environment and to the consumers. To sum up, despite continuing growth of global aquaculture, it is also worthwhile and critical to focus on developing a more sustainable industry, creating more responsible approaches based on the biological and environmental mechanisms of all related processes (FAO, 2016). One of the more sustainable options is to farm species with

low trophic level. Unlike for freshwater fish, there is limited number of cultured marine species with low trophic level. In fact, rabbit fish is a candidate.

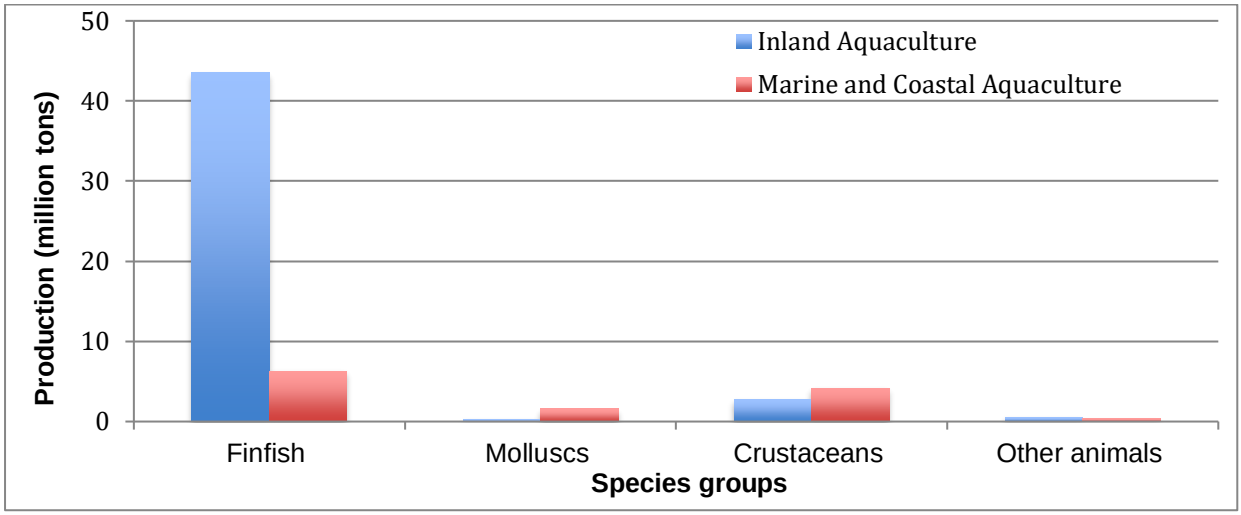


Figure 1.1. World production of farmed species from inland and marine culture in 2014. Other animals: frogs, reptiles, aquatic invertebrates (FAO, 2016).

Table 1.1. Quantity and percentage of world aquaculture production in 2014 (FAO, 2016)

Regions	Quantity (thousand tons)	Percentage (%)
Africa	1710.9	2.32
Americas	3351.6	4.54
Asia	65601.9	88.91
Europe	2930.1	3.97
Oceania	189.2	0.26
World	**73783.7**	**100**

Table 1.2. Quantity (ton) & value (US$ billion) of farmed species from inland & marine culture in 2014 (FAO, 2016)

Species groups	Quantity subtotal		Value subtotal	
	ton	% volume	value	% value
Finfish	49,861,891	67.58	99.2	61.92
Crustacean	6,915,073	9.37	36.2	22.60
Mollusk	16,113,194	21.83	21.1	13.17
Others	893,568	1.21	3.7	2.31
Total	**73,783,725**	**100**	**160.2**	**100**

1.2. The rabbit fish (*S. guttatus*) life cycle and natural food items

The females of rabbit fish produce small adhesive eggs, which then hatch into larvae. In the early stage, the larvae are planktonic and develop into a distinctive post-larval stage called the acronurus, which is characteristic for members of the suborder Acanthuroidei. In the acronurus stage, the body of the larvae is transparent and they remain pelagic beyond the outer reef or near the coastal area for an extended period before settling into the adult habitat and rapidly changing into the

juvenile form (Duray, 1998b). The juveniles and adults of rabbit fish occupy very diverse shallow water habitats (Lam, 1974), including the coral reefs (Woodland, 1979), sandy and rocky bottoms with or without vegetation (Popper and Gundermann, 1975), lagoons and river mouths (Duray, 1998b; Ayson *et al.*, 2014), and mangrove forests (Popper and Gundermann, 1975).

In the wild, the first food for rabbit fish larvae remains unknown, while the juveniles form schools in algal and sea grass beds, feeding mainly on filamentous algae (Duray, 1998b; Ayson *et al.*, 2014). The juveniles and adults are primarily herbivores and feed predominantly on different kinds of benthic algae and plants. In captivity, the brood stocks of rabbit fish become omnivorous and food on a variety of food from both vegetable and animal origin, including pellets in the culture system. In fact, rabbit fish have also been reported to consume amphipods, copepods, sponges, foraminifera, crustaceans and brittle stars, which suggest that the rabbit fish may be also opportunistic omnivores (Duray, 1998b; Ayson *et al.*, 2014).

1.3. Problems of rabbit fish (*S. guttatus*) farming in Vietnam

The rabbit fish (*S. guttatus*) can be found along the coastal area in the Central Vietnam, especially at Thua Thien Hue, Quang Nam and Binh Dinh province. Adult fish can reach up to 30 cm and weight up to 700 g. The natural spawning season is between May and June and the wild fingerlings can be collected at the river mouths three to four weeks after hatching for aquaculture. The food for grow out farming of rabbit fish are seaweed, compound diets and uneaten food from shrimp farming (Mien *et al.*, 2000; MARD, 2016b).

In aquaculture, rabbit fish is usually polycultured with the tiger shrimp (*P. monodon*) in pond at a density of 0.5 – 1 fish.m^{-2}. In the polyculture with the tiger shrimp, rabbit fish can use the residues from shrimp farming for food. After 4 – 5 months of polyculture, rabbit fish production could reach up to 0.8 – 1 tons.ha^{-1}, with up to 75% survival. The rabbit fish production in Thua Thien Hue in 2014 was approx. 1,000 tons, which was just enough for the local market (DAARD-TTH, 2014). At the moment, rabbit fish aquaculture is still totally dependent on the wild fingerling collection, which does not meet the demand of fingerlings for aquaculture (roughly 35 million fingerlings annually in Thua Thien Hue province) (MARD, 2016b), while the supply of fingerlings from wild catch was only 7 millions, which account for 20% of the demand (DAARD-TTH, 2014).

In the past, the Vietnamese Aquaculture Research Institute I, Hue University, Nha Trang University and Can Tho University have carried out researches on the artificial reproduction of rabbit fish. Unfortunately, there is no report yet on successful artificial reproduction (Mien *et al.*, 2000; Ngoan, 2006; MARD, 2016b). In conclusion, with the huge demand for rabbit fish fingerlings for aquaculture, the study on the artificial reproduction is important for the development of rabbit fish culture in Vietnam.

1.4. Objectives of this study and thesis outline

The main objective of this study is to close the life cycle of rabbit fish in captivity allowing for further experiments on the production of fingerlings at commercial level. The specific objectives involved are:

- In view of the very small mouth opening (80 µm) upon hatching, it is anticipated that rabbit fish larvae would need very small live prey. *P. similis* is a candidate live food with the body size of 83 ± 11 µm in length and 40 ± 6 µm in width. Large-scale production of rabbit fish larvae would require a considerable amount of *P. similis* individuals. Based on the size and as stated in recent reports, the microalga *Nannochloropsis oculata* (1.5-2 µm) is the most appropriate food for *P. similis*. However, the importance of the bacterial community in the diet of *P. similis* is still unknown. In this study, it is hypothesized that the growth performance *P. similis* is heavily dependent on a diet of bacteria (Chapter 3).

- The successful production of rabbit fish fingerlings is anticipated to require a specific live food feeding protocol. *B. rotundiformis* was considered to be the best starter food for rabbit fish larvae. In this study, it is hypothesized that *P. similis* will be a better starter food for rabbit fish larvae than *B. rotundiformis.* After that, specific live food and probiotic feeding protocols with *P. similis* and *B. rotundiformis* from hatching to 240 hph; followed by specific feeding protocol with *B. plicatilis* and *Artemia* from day 10 to day 25; will be developed. The data generated from this chapter will generate a protocol for rabbit fish larviculture from hatching to day 25 (Chapter 4).

- To further optimize the larviculture of rabbit fish, it is hypothesized that information on the body LC-PUFAs composition and gut MC of wild larvae entering into lagoons would be useful. For that, the LC-PUFAs composition of body tissue and the gut MC of wild larvae of rabbit fish (sampled at 3 different locations over a period of 3 years) will be investigated and compared with hatchery-reared larvae (Chapter 5).

CHAPTER 2

Review of literature

2.1. Larviculture of marine fishes in South East Asia

Aquaculture plays an important role in supplying food to many countries in South East Asia (Marte, 2003) and is expected to increase its role annually to meet the increased human consumption in the near future (FAO, 2016). Besides important high trophic level seafood *e.g.* Asian seabass (*Lates calcarifer*), orange-spotted grouper (*Epinephelus coioides*), red snapper (*Lutjanus campechanus*), cobia (*Rachycentron canadum*), red drum (*Sciaenops ocellatus*), lower trophic level seafood such as rabbit fish (*S. guttatus*), spotted scat (*Scatophagus argus*), grey mullet (*Mugil cephalus*), and milk fish (*Chanos chanos*) are considered important cultured species to fulfill the demand for food in this region. To develop sustainable aquaculture, the larviculture industry should be established adequately to supply enough fingerlings all year around for the grow out farming, in order to replace fingerlings captured from nature (Marte, 2003). In most hatcheries, marine fish larviculture is carried out in 3 – 60 m^3 canvas, concrete or fiberglass tanks (Pechmanee, 1997; Duray, 1998a; Sugama *et al.*, 2001) with stocking densities from 10 to 60 larvae.L^{-1}. The larvae are reared using the clear water (without microalgae) or green water technique (with *Chlorella vulgaris or N. oculata* added in the water) (Marte, 2003). Rotifers (*Brachionus sp.*) are commonly used as starter food during the first 2 weeks, then, they are gradually replaced by *Artemia* nauplii (Marte, 2003). Prior to feeding to the larvae, the rotifers and *Artemia* are enriched in an emulsified highly unsaturated fatty acids (HUFA) medium. Finally, the *Artemia* are gradually replaced or co-fed with compound diets from 20 dph (Bagarinao, 1986; Ordonio-Aguilar, 1995; Quinitio and Duray, 1996; Pechmanee, 1997) (Figure 2.1).

2.2. Rabbit fish larviculture

2.2.1. Biology of rabbit fish (*Siganus guttatus*)

Morphology and species identification: Rabbit fish (*S. guttatus*) (Figure 2.2) morphology was first described by Herre and Montalban (1928). The rabbit fish body is deep and compressed with a snout similar to a rabbit. The dorsal fin has 13 pungent spines, the anal fin has 7 spines, and the ventral fin has 2 spines. The skin of rabbit fish *(S. guttatus)* is leathery with smooth, small and closely adherent scales. There are few morphological differences between species in the genus *Siganus*. The orange-spotted rabbit fish differentiate from other species with a big orange spot just in front of

the tail of the fish. There are guidelines to identify the species within the rabbit fish family from Herre and Montalban (1928); Woodland (1973); Woodland and Allen (1977); Burgan and Zseleczky (1979); Burgan *et al.* (1979); Woodland (1979); Woodland and Randall (1979); Rau and Rau (1980).

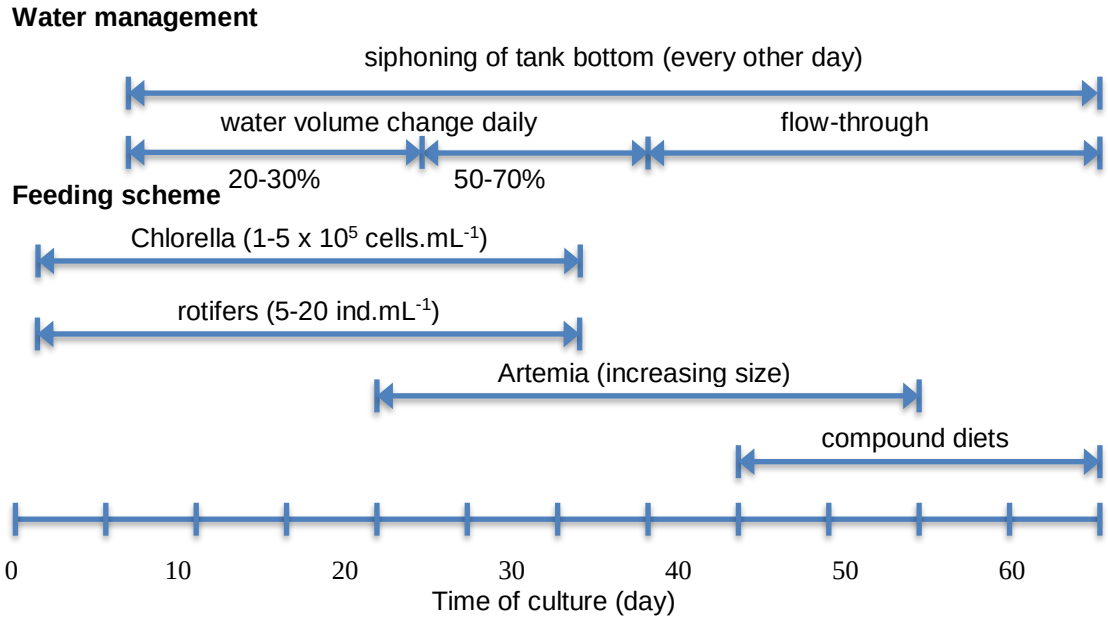


Figure 2.1. Feeding scheme and water management for rearing of milkfish (Marte, 2003)

Figure 2.2. Rabbit fish (*S. guttatus*) (Source: Ayson *et al.*, 2014)

Taxonomy: The rabbit fish belongs to the phylum Chordata, class Osteichthyes, division Halecostomi, order Perciformes, family Siganidae, genus *Siganus* and species *S. guttatus* Bloch, 1787. The common names are orange-spotted rabbit fish or golden rabbit fish (Day, 1888; Starks, 1907; Woodland, 1973; Woodland and Allen, 1977).

Geographic distribution and habitats: Previous studies described that rabbit fish appeared in various areas around the world, such as Indian Ocean, Pacific Ocean, the Eastern and Western of the Indo-Malay area, Western Indian Ocean, Red Sea, Mediterranean Sea and Northwestern Australian Province (Day, 1888; Starks, 1907; Woodland, 1973; Woodland and Allen, 1977; Woodland, 1979; 1983) (Figure 2.3).

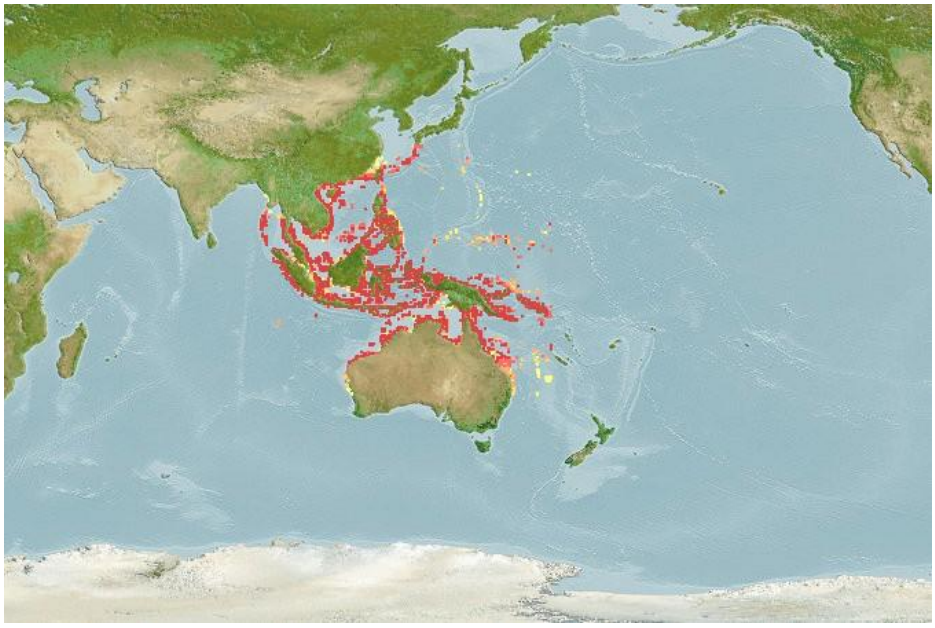


Figure 2.3. Geographic distribution of rabbit fish (*S. guttatus*) (red spots) (Source: FishBase)

Gundermann *et al.* (1983) reported that rabbit fish (*S. guttatus*) school at some stage in life. They could adapt to a wide variety of salinity and temperature (Woodland and Allen, 1977). The rabbit fish (*S. guttatus*) larvae at early stages are pelagic and commonly found close to the water surface above the coral reef (Johannes, 1978). Later, the larvae migrate to the lagoons and river mouths (Munro, 1967), mangrove forests and swamps (Alcala, 1979; Gundermann *et al.*, 1983), sandy or rocky bottom coast (Popper and Gundermann, 1975; Hasse, 1977) or stay at the coral reef (Lam, 1974; Woodland, 1979; Woodland and Randall, 1979).

Reproductive characteristics of rabbit fish (*S. guttatus*): The morphology of male and female rabbit fish is different. Firstly, the body of male rabbit fish is smaller, more elongated and the abdomen is plumper than the female (Manacop, 1959; Helfman, 1968). In addition, the anus aperture of the female is bigger than the male. Based on histological evidence, Juario *et al.* (1985) and Soletchnik (1984) observed that the hatchery-bred male *S. guttatus* matured at 10 months (19 cm total length, 200 g), while the female matured at 12 months (21.5 cm total length, 260 g). Based on the

morphology of the ovary/testis, there are 6 stages of rabbit fish gonadal development according to Lavina (1975), and based on the size of the gonad, the rabbit fish gonadal development is divided into 7 stages (Alcala and Alcazar, 1979) (Table 2.1).

Table 2.1. Gonadal development of *S. guttatus*, according to Lavina (1975), Alcala and Alcazar (1979)

Stage	Lavina (1975)	Alcala and Alcazar (1979)
1	Immature gonad	14 – 70 mm (the chromatin stage of nucleolus, the early perinucleolar stage of oocytes)
2	Developing virgin or recovering spent (ovary or testis)	14 – 238 mm (the late perinucleolar and yolk vesicle oocytes)
3	Ovary/testis mature in progress	56 – 350 mm (the vesicle stage of yolk and primary stage of oocytes)
4	Ovary/testis mature in captivity	210 – 364 mm (the secondary and tertiary stage of oocytes)
5	Ovary/testis ripe and ready to spawn	266 – 406 mm (the oocytes is mature in captivity)
6	Unable to spawn, spending stage	336 – 420 mm (eggs are ripe and ready to spawn)
7		14 – 70 mm (after spawning rest or spend after unable to spawn)

Soletchnik (1984) observed that the vitellogenesis cycle of *S. guttatus* is completed within 27 – 28 days and the vitellogenesis cycle is largely dependent on the quantity and quality of diet. For example, females fed 43% protein pellets could spawn consecutively for 11 months (Soletchnik, 1984). Hara *et al.* (1986c) also reported that females fed the fatty diets containing lecithin and cod liver oil could spawn for at least 4 consecutive months. Soletchnik (1984) and Hara (1987) described that a 400 g female with 13.8 gonadosomatic index (GSI) can spawn 800,000 eggs.

2.2.2. Larviculture of rabbit fish

Spawning activity: The natural spawning of captive *S*. *guttatus* has been observed monthly in 0.5 – 6 m^3 canvas or fiberglass tanks with little water change at 2 – 3 days after the first quarter of the lunar cycle all year round (Soletchnik, 1984; Juario *et al.*, 1985; Hara *et al.*, 1986a; Hara *et al.*, 1986c). In addition, the female rabbit fish can be induced to spawn by HCG hormone or environmental change (changing the water level simulating tidal regime). The spermiation response of mature male rabbit fish is induced using LHRHa (Lam, 1974; Bryan, 1975a; Burgan and Zseleczky, 1979; Anon, 1983; Hara *et al.*, 1986c; Ayson, 1989). Rabbit fish spawning activity is strongly synchronized with moonlight cycle (Takemura *et al.,* 2004). During spawning season,

the adults matured by the new moon. Spawning activity was reported between the first quarter of the moon and the full moon, including Vietnam (Hara *et al.*, 1986c; Takemura *et al.*, 2004; Ngoan, 2006; Park *et al.*, 2007; Ikegami *et al.*, 2014). At present, the spawning grounds or egg distribution of rabbit fish have not been reported. It is also very difficult to obtain either eggs or early stage larvae from nature (Manacop, 1937; Hasse, 1977).

The hatching of eggs, larval development and larval rearing of rabbit fish: After spawning, the egg diameter of rabbit fish range between 0.42 and 0.70 mm, demersal and adhesive to the substrate such as plants, sea grass and rocks at the bottom of the sea. The stages of development of the fertilized eggs were described in Figure 2.4 (Manacop, 1937; Fujita and Masaaki, 1954; Lam, 1974; Westernhagen *et al.*, 1974; Hasse, 1977; Burgan and Zseleczky, 1979; Luchavez and Carumbana, 1982; Leis and Rennis, 1983; Avila, 1984; Juario *et al.*, 1985; Hara *et al.*, 1986b). In the optimal water temperature (22-30°C), it took 18-35 h for fertilized eggs to hatch (Westernhagen, 1973; Lam, 1974; Westernhagen, 1974; Westernhagen *et al.*, 1974; Luchavez and Carumbana, 1982). There is no report on the distribution of rabbit fish eggs in the wild, presumably because of the difficulty in sampling demersal and adhesive eggs (Duray, 1998b). Details of the length development of rabbit fish larvae are indicated in Figure 2.5. After hatching (0 hph, stage 1), the larvae distribute at the water surface. The length of the larvae is 1.5 – 2.6 mm with a straight intestine, closed mouth and eyes (Duray, 1998b). The mouth opens at 30.5 hph (Ordonio-Aguilar, 1995). The larval yolk volume is small (0.70×0.24 mm), formed together with two oil globules. From 6 hph (stage 2) to 13 hph (stage 3), the larvae use the yolk energy for a rapid growth from 2.15 to 2.80 mm in length. From 24 hph (stage 4, 2.80 mm) to 48 hph (stage 6, 2.75 mm), the larvae show slow or even negative growth due to the rapid decrease of yolk volume. Once the larvae can consume the rotifers (from day 3 onwards), the length increased quickly to 7.92 mm at 192 hph (Figure 2.5). At hatching, the rabbit fish larvae are pelagic and very fragile. There are 24-myotomes and melonaphores distributed around the snout, yolk and globules, and near the ventral side of the larvae. The cupulae are observed on free neuromasts at 6 hph and disappear at 39 hph. The presence of cupulae made the larvae very sensitive to handling and difficult to catch by pipette. In case of handling, the larvae would die (Hara *et al.*, 1986c; Kohno *et al.*, 1988). Most importantly, the head armor of rabbit fish protects the larvae

against predators through increasing flotation and body size to deter predators, and hence, improve larval survival (Hara, 1987). Kohno et al. (1986) described the development of morphology in rabbit fish based on body length. In detail, the dorsal and ventral fin rays of S. guttatus larvae appear and develop around 4.0 mm. The premaxillae appear at around 4.1 mm. The flexion of the notochord is completed at 6.5 mm. Fin rays appear at 8.0 mm. The jaw and pharyngeal teeth appear and develop from 4.0 - 4.5 mm. The availability of jaw and pharyngeal teeth allow the larvae to change gradually the feeding habit at 7.0 – 8.0 mm (Table 2.2 and 2.3). On day 12, rabbit fish larvae start to eat on algae growing on the tank walls. The larval behavior becomes more aggressive from day 15, and cannibalism starts through biting on other larval tails or eyes (Juario *et al.,* 1985). On day 18, larvae swim toward the tank bottom and stay deeper in the water column. Schoaling begins at day 23 (Hara 1987) (Table 2.3). The complete larvae morphology, behavioral development and feeding habits are described in the Table 2.2 and 2.3. The *S. guttatus* larvae approach the juvenile stage (22.0 mm after 45 days from hatching) when they obtain the full complement of spines and fin rays of the adults. Juveniles also resemble the adults in body colour and shape (Duray, 1998a).

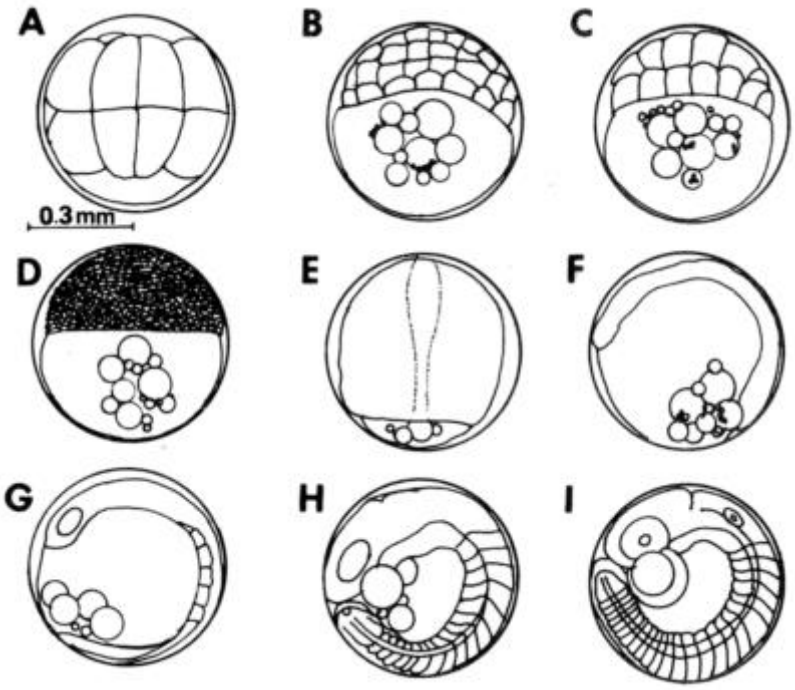


Figure 2.4. Embryogenesis of rabbit fish (Hara *et al.*, 1986b); (A): 8-cell stage (0.5 h post fertilization – hpf); (B): 32-cell stage (1 hpf); (C): morula stage (1.5 hpf); (D): blastula stage (2 hpf): (E): embryonic shield stage (6 hpf); (F): development of embryonic body stage (7 hpf); (G): 6-myomere stage (8 hpf); (H): 16-myomere stage (11 hpf); (I): 24-myomere stage (13 hpf).

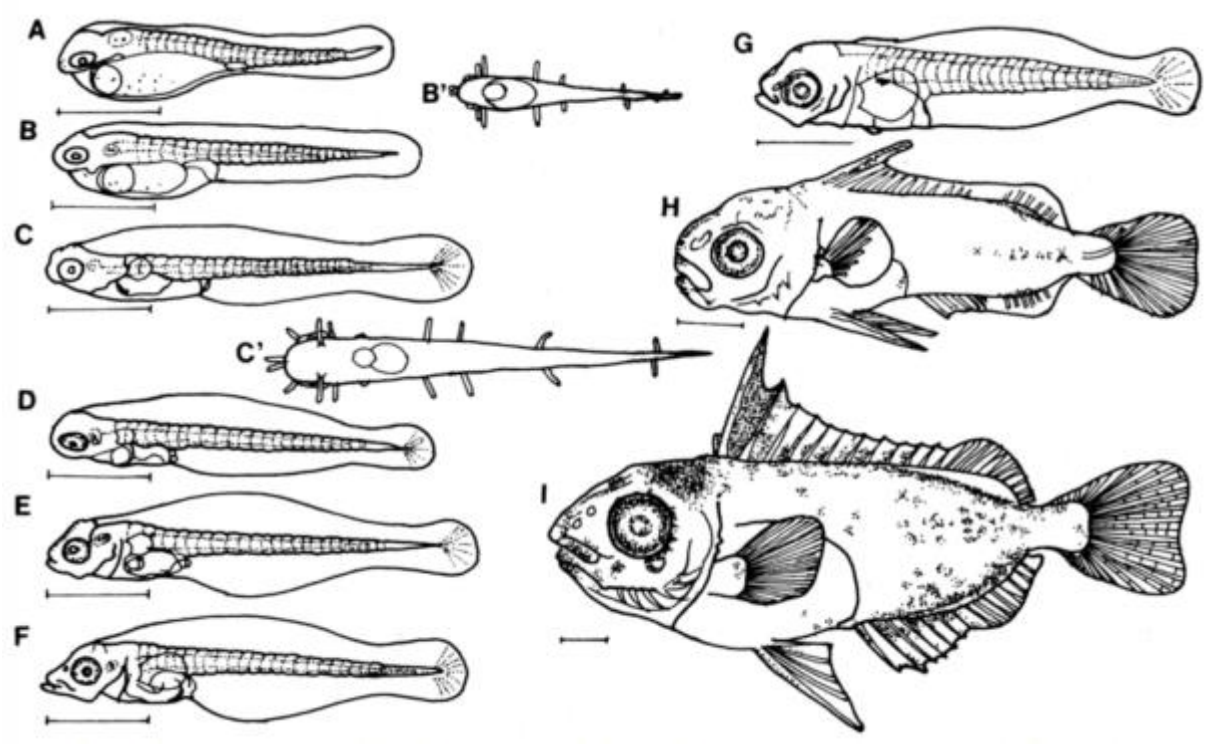


Figure 2.5. The length of the *S. guttatus* larvae in 9 stages of development (Hara *et al.*, 1986b): stage 1 (A): newly hatched larvae (0 hph), 1.72 mm total length (TL); stage 2 (B): 6 hph, 2.15 mm TL; (B'): ventral side of larvae at stage 2; stage 3 (C): 13 hph, 2.80 mm TL; (C') ventral side of larvae at stage 3; stage 4 (D): 24 hph, 3.00 mm TL; stage 5 (E): 39 hph, 2.84 mm TL; stage 6 (F): 48 hph, 2.75 mm TL; stage 7 (G): 192 hph, 4.40 mm TL; stage 8 (H) 312 hph, 7.92 mm TL; stage 9 (I): 408 hph, 13.07 mm TL. Bar scale: pictures from A to F: 0.5 mm; pictures from G to I: 1.0 mm.

Table 2.2. The morphology of *S. guttatus* larvae from hatching to 9.6 mm.

Length (mm) or age (hph)	Morphology	References
6 – 39 hph	Neuromasts containing cupulae	(Hara *et al.*, 1986b; Pantoja and Kadowaki, 1988)
30.5 hph	Mouth opening (80 µm)	(Ordonio-Aguilar, 1995)
2.6 mm	The beginning of rheotaxis	(Hara, 1987)
2.7 mm	The beginning of phototaxis	
3.9 mm	The development of dorsal and ventral fins	(Kohno *et al.*, 1986)
4.0 – 4.5 mm	The development of jaw and pharyngeal teeth	
4.1 mm	The appearance of premaxillae	
6.5 mm	The premaxillae occupied in most of the gape	
7.0 – 8.0 mm	The feeding habit is similar to the adult	(Hara, 1987)
8.0 mm	The complete development of fin rays. The fin rays are morphologically similar to adult	
9.6 mm	The phototaxis disappeared	

Table 2.3. The behavior/feeding habits of *S. guttatus* larvae from hatching to day 24.

Age (dph)	Behavior/Feeding habits	References
1	The larvae grew very fast in length using nutrients from yolk Swimming with head down, often going up and down quickly in the water column. Staying and resting near the water surface	(Westernhagen, 1973; 1974; Bryan, 1975b; Kohno *et al.*, 1986)
2	The larvae grew slower as the yolk sac was nearly exhausted. The larvae started some initial feeding on small rotifers. Rabbit fish larvae are relatively diligent feeders. Learning how to feed on the wall sides of the rearing tanks	(Luchavez and Carumbana, 1982; Kohno *et al.*, 1986; Ordonio-Aguilar, 1995)
3	The larvae grew slowly using nutrients from reserved energy (remaining yolk, oil globules) and exogenous food	(Kohno *et al.*, 1986)
4	The larvae grew slowly using nutrients from reserved energy (oil globules) and exogenous food	(Juario *et al.*, 1985)
5-6	The larval growth accelerated and swimming actively based on energy from exogenous food	(Juario *et al.*, 1985)
>6	The food consumption increased rapidly and the larvae grow rapidly	(Juario *et al.*, 1985)
12	Eating predominantly on algae growing on wall sides of the rearing tanks	(Juario *et al.*, 1985)
15	The larvae start swimming erratically exhibiting some cannibalism. The tail and eyes of smaller larvae are bitten by bigger ones.	(May *et al.*, 1974; Juario *et al.*, 1985)
18	The larvae start to migrate to the bottom of the tanks	
23	The larvae start metamorphosis. Their appearance looks similar to the adult after metamorphosis. They swim actively up and down the water column searching for food	(Hara, 1987)
24	Body color turns from transparent to brown similar to the adult	(Hara *et al.*, 1986b)
24	Starting migration from offshore to the coastal area. Under hatchery conditions, the migration speed of larvae is 58.2 cm per minute at 60 hph, 70.6 cm per minute at 156 hph day 6 1/2	(Gundermann *et al.*, 1983; Kishimoto, 1984; Duray, 1998b)

Environmental parameters and requirements in the early stages of rabbit fish (*S. guttatus*) development: The eggs of rabbit fish (*S. guttatus*) are highly tolerant to a wide salinity range, where over 90% of the eggs could hatch at salinities from 3 to 71 $g.L^{-1}$ and the optimum salinity for spawning and hatching are 24 to 32 $g.L^{-1}$ (Young and Dueñas, 1993). Hara *et al.* (1986b) also reported that in the range of 20 – 32 $g.L^{-1}$, salinity did not affect the incubation and hatching of the eggs. In the salinity range of 14 – 37 $g.L^{-1}$, more than 50% fertilized eggs could be achieved. The high tolerance to salinity is also observed in the rabbit fish over 21 days old. They survived in salinity ranging from 2 $g.L^{-1}$ to 55 $g.L^{-1}$. In freshwater conditions (0 $g.L^{-1}$), the larvae died after 48 – 72 h (Westernhagen, 1973; 1974; Westernhagen *et al.*, 1974; Carumbana and Luchavez, 1979). Hara *et al.* (1986c); Kohno *et al.* (1988) reported that rabbit fish (*S.*

guttatus) larvae can be reared at 22 to 30°C, and the highest survival of larvae is obtained at a temperature of 22 to 26°C. Within the optimal range of temperature, the metabolism rate of rabbit fish larvae is highest at 30°C. At a temperature of 30°C, the pigmentation of the larval eyes is observed 3 h earlier than at 27°C. The larvae could endure a low level of dissolved oxygen of 0.7 $mg.L^{-1}$. The optimal DO level for larval rearing is above 4 $mg.L^{-1}$ (Ben-Tuvia *et al.*, 1972; Tobias, 1976; Carumbana and Luchavez, 1979). Besides, Duray and Kohno (1988) suggested that continuous light would improve the survival of first-feeding rabbit fish (*S. guttatus*) larvae.

2.3. Types of food for the early stages of marine fish larvae

2.3.1. Microalgae

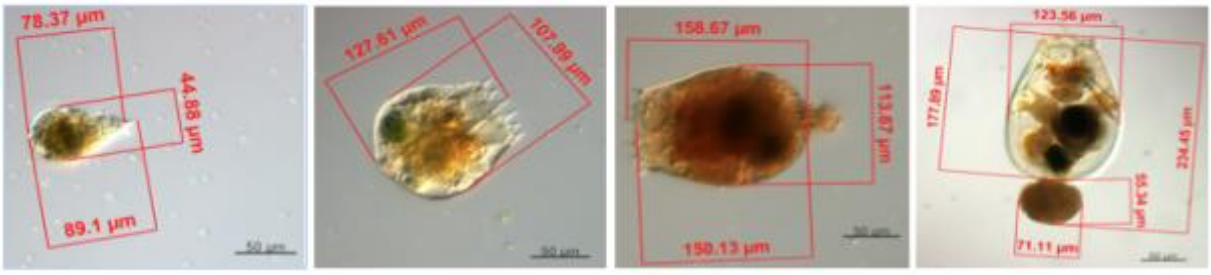
Microalgae are the first and an important link in the marine food chain, as primary producer, due to the ability to use sunlight for organic molecules' synthesis. In marine fish larviculture, microalgae are used as an indirect food at the early larval stage. Species such as *N. oculata, Tetraselmis sp., C. vulgaris, Isochrysis galbana, Pavlova sp*. had important roles and have been documented to facilitate transfer of PUFAs, amino acids and vitamins from microalgae through zooplankton to fish larvae (Makridis and Olsen, 1999; Chakraborty *et al.*, 2007; Hemaiswarya *et al.*, 2011). Additionally, microalgae (*N. oculata* and *C. vulgaris*) are also used in the larval rearing for the green water technique, in which microalgae are used directly in the larval tanks. The green water technique is reported to improve the survival of fish larvae (Marte, 2003), hence, it is widely applied in the marine fish rearing protocols (Yúfera and Lubián, 1990; Reitan *et al.*, 1997). Moreover, many reports indicated that the larval quality is improved when adding microalgae to the rearing water, and it is observed that some larvae consumed microalgae directly (Moffatt, 1981). Finally, the addition of microalgae to the rearing water "enhanced" the MC in the water and in the larval gut (Nicolas *et al.*, 1989; Reitan *et al.*, 1997; Skjermo and Vadstein, 1999; Olsen *et al.*, 2000).

2.3.2. Rotifers

Rotifera are one of the smallest metazoans of which over 2200 species have been described. The rotifer body size used in larviculture ranges from 35 to 350 µm, an appropriate size of prey to start feeding after the yolk sac of many marine fish species is exhausted (Yufera *et al.*, 1984; Polo *et al.*, 1992; Olsen *et al.*, 2000). Although *B.*

plicatilis was first found as a pest in a pond, Japanese researchers (around 1970's) soon realized that they could be used as a suitable live food organism for the early larval stages of marine fish since the 1970s. The successful use of rotifers in the commercial hatchery operations of the red sea bream (*Pagrus major*) encouraged investigations in the development of large-scale culture techniques of rotifers (Fukusho and Iwamoto, 1981; Fukusho, 1989; Dhert *et al.*, 1995; Lavens and Sorgeloos, 1996; Hirata *et al.*, 1998). The *B. plicatilis* species complex is a group of euryhaline rotifers in the *Brachionidae* family. They are raised in the aquaculture industry as food for most marine fish larvae. A simple classification is still used in aquaculture based on three different morphotypes, namely super small (SS-type, 90 – 110 µm), small (S-type, 100 – 120 µm) and large (L-type, 130 – 340 µm) rotifers (Dhert *et al.*, 1995; Hagiwara *et al.*, 1995; Lavens and Sorgeloos, 1996; Hagiwara *et al.*, 2001) (Figure 2.5), although DNA-based characterization has been developed (Gómez and Carvalho, 2000; Papakostas *et al.*, 2006). Currently, they are classified into 15 species (Segers, 1995; Ciros-Pérez *et al.*, 2001; Gómez *et al.*, 2002; Suatoni *et al.*, 2006; Fontaneto *et al.*, 2007). Apart from the appropriate body size, rotifers have a lot of advantages for marine fish larviculture, including (i) slow swimming; (ii) high reproduction rate by parthenogenesis; (iii) filter feeding on a wide variety of foods; (iv) tolerance to extreme culture conditions (low oxygen level, salinity changes…) and handling (harvesting); (v) the ability to bioencapsulate various kinds of nutrients, such as protein, HUFA, ARA and vitamins; and lastly (vi) they can be year-round cultured, making the hatchery independent of the live food supply, avoiding being dependent on external supplies. The main rotifers cultured for fish mariculture include the species/genotypes within the euryhaline *B. plicatilis* complex (Yufera, 1982; Fukusho and Okauchi, 1983; Snell and Carrillo, 1984; Gómez and Serra, 1995) such as Nevada, Cayman and Austrian strain (Papakostas *et al.*, 2006; Dooms *et al.*, 2007; Baer *et al.*, 2008). The SS-type is the smallest *Brachionus sp.* used in aquaculture and is classified as *B. rotundiformis* (Segers, 1998; Kotani *et al.*, 2005; Fontaneto *et al.*, 2007). Due to its smaller size, *B. rotundiformis* is commonly used as starter food for fish species with a small mouth gape. However, feeding mixed stages of *B. rotundiformis* is ineffective or unsuitable for the larvae of several marine fishes with even a smaller mouth, including some species of groupers (Kohno *et al.*, 1997; Glamuzina *et al.*, 1998), angelfishes (Olivotto *et al.*, 2006) and wrasse (Sugama *et al.*, 2004). Recently, Wullur *et al.* (2009) found a minute euryhaline rotifer, namely *P. similis*, with a body size of 83 ± 11 µm in length and 40 ±

6 µm in width, which has the potential to be the first food for small-sized mouth marine fish larvae (Figure 2.6). The optimal temperature for *P. similis* culture is 30 – 35 °C and this rotifers can grow well at a wide salinity range (2 – 25 $g.L^{-1}$). The life span, generation time and reproductive period are 2.4 – 4.7 days, respectively depending on culture conditions (such as temperature). The fecundity is 4.3 – 7.8 $female^{-1}$ (Wullur *et al.*, 2009). Many reports recently stated that *P. similis* is the most suitable live food for the first feeding stage of a variety of marine species with a very small mouth/special esophagus at opening, including the seven-band grouper *E. septemfasciatus*, the rusty angelfish *Centropyge ferrugata*, the humphead wrasse *Cheilinus undulatus* and the Japanese eel *Anguilla japonica* (Wullur *et al.*, 2009; 2011; Hirai *et al.*, 2012; Hirai *et al.*, 2013; Wullur *et al.*, 2013; Hagiwara *et al.*, 2014).



A B C D

Figure 2.6. Rotifers used in fish larviculture. A. *P. similis*. B. *B. rotundiformis*. C&D. *B. plicatilis* (S&L) (own measurements)

2.3.3. Copepods

Copepods are one of the main food for most marine fish larvae in nature. Copepods belonging to the orders Calanoida, Harpacticoida and Cyclopoida are cultured commercially and used in the hatchery for the feeding of fish larvae, due to its small size at the nauplius stage (Payne and Rippingale, 2001; Stottrup, 2003; Lee *et al.*, 2008). For example, the harpacticoid *Schizopera elatensis, Tisbe holothuriae* and *Tisbentra elongata* nauplius size are 50, 55 and 150 µm, respectively (Lavens and Sorgeloos, 1996). Many reports indicated that larvae performed better when fed copepod nauplii, compared to rotifers and Artemia, especially for Atlantic halibut and Atlantic cod larvae (Næss *et al.*, 1995; Støttrup *et al.*, 1998; Shields *et al.*, 1999; Hamre *et al.*, 2002; Rajkumar and Vasagam, 2006) due to its high nutritional content (Moren *et al.*, 2006; Hamre *et al.*, 2008; van der Meeren *et al.*, 2008). For example, the EPA, DHA and (n-3)HUFA levels in *Tisbe* nauplii fed *Dunaliella* are high, (around 3.5, 9.0 and 15% of body composition, respectively) (Lavens and Sorgeloos, 1996) and the EPA and DHA levels of *A. tonsa* nauplii newly hatched from eggs was 15.5

and 34.8%, respectively) (Støttrup *et al.*, 1999). However, the use of copepod nauplii in the hatchery is not as popular as rotifers and other zooplankton, mainly because of its costly culture protocol. As a result, copepod nauplii are only fed to the larvae in experimental scale or in hatcheries located near the natural harvesting grounds. In case copepods are available in sufficient amounts, they could be used as food to the larvae (Conceição *et al.*, 1997; Toledo *et al.*, 1999), especially for larvae with small yolk sac volume and small mouth size at opening, such as groupers and red snapper (Toledo *et al.*, 1999; Ogle *et al.*, 2005; Toledo *et al.*, 2005).

2.3.4. *Artemia*

Artemia are commonly used as live food in larviculture of marine fishes worldwide. In most fish species, the larvae need to food on rotifers before weaning to *Artemia*. However, in some species with relatively large larvae (salmon) (Kim *et al.*, 1996), *Artemia* can be used as the first and only live food until juvenile stage. *Artemia* cysts are famous for its ease of handling, mass hatching and production. However, newly hatched *Artemia* nauplii contain very low DHA levels, and are expensive (Van Stappen, 1996b; a; Lavens and Sorgeloos, 2000; Fernández, 2001). Similar to rotifers, the metanauplius stage of *Artemia* are continuous non-selective filter-feeding organisms and could be enriched with nutrients (HUFAs and vitamins) through bio-encapsulation (Lavens and Sorgeloos, 1996) prior to feeding to the fish larvae.

2.3.5. Micro-formulated diets

The larviculture of marine fishes is largely dependent on live food, such as rotifers and *Artemia* (Liao *et al.*, 2001). However, the production of live food is relatively costly and a possible source of bacteria-contamination to the larval rearing system (De Pauw *et al.*, 1983; People Le Ruyet *et al.*, 1993; Skjermo and Vadstein, 1999; Salvesen *et al.*, 2000; Villamil *et al.*, 2003; Rawlings *et al.*, 2007; Gugliandolo *et al.*, 2008; Conceição *et al.*, 2010). Since 40 years, micro-formulated diets are produced and are considered as one of the alternatives for the live food in larviculture. Up to date, there are 6 different types of micro-formulated diets: microbound particles (Teshima *et al.*, 1982; Barrows *et al.*, 1993; Holt, 1993; López-Alvarado *et al.*, 1994; Canavate and Fernández-Dıaz, 1999; Barrows and Hardy, 2000; Baskerville-Bridges and Kling, 2000; Önal and Langdon, 2000), cross-linked protein-walled capsules (Gatesoupe *et al.*, 1977; Kanazawa *et al.*, 1982; Teshima *et al.*, 1982; Jones *et al.*, 1984), lipid-walled

microcapsules (Langdon and Siegfried, 1984), lipid spray beads (Langdon and Buchal, 1998), liposomes (Ostro, 1987; Kulkarni *et al.*, 1995; Coutteau and Sorgeloos, 1997) and complex particles (Hayward *et al.*, 1987; Villamar and Langdon, 1993; Baskerville-Bridges and Kling, 2000; Langdon, 2000). Despite the advantages and development of micro-formulated diets, there are still a number of difficulties for the wide application of micro-formulated diets in the larval rearing. Firstly, some digestive enzymes for the digestion of micro-formulated diets, such as lipase and amylase, are still absent in the larval gut (Blaxter *et al.*, 1983; Cataldi *et al.*, 1987; Munilla-Moran *et al.*, 1990; Kjørsvik *et al.*, 1991; Infante *et al.*, 1997; Kolkovski *et al.*, 1997; Hamlin *et al.*, 2000; Cahu and Infante, 2001; Kolkovski, 2001). Secondly, micro-formulated diets normally contain denatured, insoluble or slightly soluble proteins and complex carbohydrates that are very different from those of living cells, which commonly contain dissolved nutrients (Langdon, 2000). Thirdly, this reduces the digestion efficiencies of micro-formulated diets in the larval gut (Kolkovski *et al.*, 1993). Lastly, the micro-formulated diets could contain a high concentration of free amino acids, however, this concentration declines rapidly in water due to very high leakage rates (Rust *et al.*, 1993; López-Alvarado *et al.*, 1994; Rust, 1995; Cahu and Infante, 2001). By contrast, live food, such as *Artemia*; contain high level of dietary free amino acids, which could be delivered easily to the larvae without any leakage (Dabrowski and Rusiecki, 1983; Webb and Chu, 1983; Frolov *et al.*, 1991; Roeck-Holtzhauer *et al.*, 1993; Fyhn *et al.*, 1995; Næss *et al.*, 1995; Helland *et al.*, 2000). Moreover, some studies reported that the tissues of live food had better smell and taste because of free amino acids, resulting in the increased releasing of digestive enzymes and appetite of larvae (Munilla-Moran *et al.*, 1990; Cahu and Infante, 1995; Infante *et al.*, 1997; Kolkovski *et al.*, 1997; Cahu *et al.*, 1998; Lazo *et al.*, 2000; Nikolaeva and Kasumyan, 2000; Koven *et al.*, 2001). In addition, there is little knowledge about the real nutritional requirements of marine fish larvae. Therefore, some essential nutrients might be absent or leaking rapidly, such as vitamin C and HUFAs, resulting in the poor growth of fish larvae when fed micro-formulated diets (Slinger *et al.*, 1979; Goddard, 1995). Finally, the larvae feed visually and predominantly on moving live food of the right size in a tank with the appropriate color (Ostro, 1987; Mukai *et al.*, 1994; Pankhurst, 1994; Job and Bellwood, 1996; Roo *et al.*, 1999; Cox and Pankhurst, 2000; Cahu and Infante, 2001). As a result, the movement of artificial particles need to be similar to live preys, and settlement losses need to be reduced in order to enhance the feeding efficiencies of the fish larvae

(Backhurst *et al.*, 1988). Although micro-formulated diets had over 40 years of development, there are still few reports on micro-formulated diets totally replacing live food in larval rearing. Hence, depending on the fish species, micro-formulated diets could be co-fed with live food with different ratios in the first feeding stages, prior to weaning into a total replacement of live food in later stages of larval rearing.

2.4. The importance of small food size in the first feeding stage of rabbit fish (*S. guttatus*)

Live food such as rotifers or *Artemia* has been considered as the most convenient food for marine fish larvae at early stage. In terms of prey size, it was suggested that the effective dimensions of prey for most marine fishes ranges from 20 to 70% of the mouth size (Planas and Cunha, 1999; Yúfera and Darias, 2007; Rønnestad *et al.*, 2013). Rabbit fish larvae may require a starter food of 44 – 140 µm. The rotifers of *Brachionus* genus are commonly used for most marine fish larvae at first feeding (Watanabe and Kiron, 1994). However, the *Brachionus sp.* (90 – 340 µm) (Dhert *et al.*, 1995; Hagiwara *et al.*, 1995; Lavens and Sorgeloos, 1996; Hagiwara *et al.*, 2001) is bigger than the preferred food size of first feeding siganid larvae (Hara *et al.*, 1986b). Hence, it is necessary to provide a smaller food at the first feeding stage (Hara *et al.*, 1986a; Ayson, 1989; Diani *et al.*, 1990; Duray, 1998b). Alternative first feeding food has been studied for the siganids, consisting of bacteria, microalgae and oyster D-larvae (Juario *et al.*, 1985; Bagarinao, 1986; Kohno *et al.*, 1986; Kohno *et al.*, 1988).

The first nominee, bacteria (0.2 µm in width, 2 – 8 µm in length), is too small and not visual for fish larvae to capture. Secondly, microalgae (from 1.5 µm in width and 14 µm in length, are too small to be retained by the larvae gill rakers, as they are not filter feeders. In the green water technique, microalgae are found in the gut, but some reports indicated that the microalgae are there through rotifers or ingested through drinking (Bagarinao, 1986; Kohno *et al.*, 1986; Kohno *et al.*, 1988). Next, the bivalve larvae, for example, the oyster D-larvae (55 – 75 µm) would fit the size. However, besides the inconsistent supply, the oyster D-larvae formed shells within 6 h at 30°C and started settling to the tank wall, as a result, the residue of oyster D-larvae would destroy the culture tanks. Therefore, the use of oyster D-larvae in larval rearing is limited to date (Juario *et al.*, 1985). When rotifers (*Brachionus sp.*) were fed to the rabbit fish larvae at first feeding, Kohno *et al.* (1988) stated that feeding of rotifers

could only start when the *S. guttatus* had a mouth size of 200 µm, which is at 55.5 h after hatching. The ingested rotifer had a width of 125 µm and is by far the most suitable food to larvae of *S. guttatus*, at that stage. In current practice of rabbit fish larviculture, newly hatched rotifers (*Brachionus sp.*) are collected by straining the rotifer culture through 80 or 100 µm mesh size net prior to feeding to the larvae. However, this is a wasteful way of feeding, as only 10% of the rotifers can pass through the 80 µm net and furthermore, the rotifers subsequently, grow quickly in the larval tank (Hara *et al.*, 1986a). When the fry grow bigger, another suitable live food species is *Artemia sp.* (500 – 600 µm).

2.5. The roles of LC-polyunsaturated fatty acids (LC-PUFAs) in fish

LC-PUFAs are fatty acids with 18 or more carbons, which can be categorized into two main families — ω6 (n-6) and ω3 (n-3) — depending on the position of the first double bond from the methyl end group of the fatty acid. The main n-3 LC-PUFA in food sources are *α*-linolenic acid (ALA) (18:3 Δ9, 12, 15), docosahexaenoic acid (DHA) (22:6 Δ4, 7, 10, 13, 16, 19), eicosapentaenoic acid (EPA) (20:5 Δ5, 8, 11, 14, 17), and docosapentaenoic acid (DPA) (22:5 Δ7, 10, 13, 16, 19), and n-6 LC-PUFA include linoleic acid (LA) (18:2 Δ9, 12) and arachidonic acid (AA) (20:4 Δ5, 8, 11, 14) (Venegas-Calerón *et al.*, 2010). Most fish have no ability to synthesize the long chain polyunsaturated fatty acids (PUFAs) from C18 fatty acids (Watanabe *et al.*, 1984a; Watanabe *et al.*, 1984b; Sargent and Henderson, 1995; Izquierdo *et al.*, 2001; Suloma and Ogata, 2011), except some species (examples in Table 2.4). The long-chain PUFAs biosynthesis from C18 (linolenic and linoleic fatty acids) contains a series of sequential reactions by multiple fatty acyl desaturases (Fads), *e.g.* Δ4, Δ5, Δ6, Δ6/Δ5, Δ6/Δ8, Fads and elongase of very long-chain fatty acid (Elovl) enzymes, *e.g.* Elovl2, Elovl4 and Elovl5 (Agaba *et al.*, 2004; Li *et al.*, 2010; Monroig *et al.*, 2011). The capability of long-chain PUFAs biosynthesis is different amongst fish species (Table 2.4), and is determined by the availability of biosynthetic enzymes in their body (Castro *et al.*, 2016). Generally, freshwater teleosts possess the ability to synthesise their own long-chain HUFA from C_{18} fatty acids. By contrast, marine teleosts lack this ability due to an apparent deficiency of the Δ5-desaturase required for the elongation and desaturation of these C_{18} precursors to AA and EPA (Sargent, 1995). However, the Δ6/Δ5 Fads enzyme, which is critical for the biosynthesis of the PUFAs, was found in rabbit fish, hence, they are unique amongst marine teleosts having the ability to

synthezise all long chain PUFAs from C18. The fatty acyl desaturases (Fads) and the elongases of very long-chain fatty acids (Elovl) in fish are presented in Table 2.4.

Table 2.4. The summary of the fatty acyl desaturases (Fads) and the elongases of very long-chain fatty acids (Elovl) in marine fish.

Species	Reported activities	References
Rabbit fish *(S. canaliculatus)*	Δ4, Δ6/Δ5 Fads, Elovl4, Elovl5	Li *et al.* (2010); Xie *et al.* (2016); Monroig *et al.* (2012)
Zebra fish *(Danio rerio)*	Δ4, Δ5, Δ6, Δ8 Fads, Elovl4, Elovl5, Elovl2	Hastings *et al.* (2001); Agaba *et al.* (2004); Monroig *et al.* (2009); Monroig *et al.* (2010a)
Atlantic salmon (*Salmo salar*)	Δ6, Δ8 Fads, Elovl2, Elovl4, Elovl5	Agaba *et al.* (2005); Zheng *et al.* (2005); Morais *et al.* (2009); Monroig *et al.* (2010b); Carmona-Antoñanzas *et al.* (2011)
Cobia (*Rachycentron canadum*)	Δ6, Δ8, Elovl4, Elovl5	Zheng *et al.* (2009); Monroig *et al.* (2011)
Gilthead sea bream (*S. aurata*)	Δ6, Δ8 Fads, Elovl5	Seiliez *et al.* (2003); Zheng *et al.* (2004); Agaba *et al.* (2005)
Turbot (*Psetta maxima*)	Δ6, Δ8 Fads, Elovl5	Zheng *et al.* (2004); Agaba *et al.* (2005)
Atlantic cod (*Gadus mordua*)	Δ6, Δ8 Fads, Elovl5	Tocher *et al.* (2006); Agaba *et al.* (2005)
Common carp (*Cyprinus carpio*)	Δ6 Fad, Elovl5	Zheng *et al.* (2004); Agaba *et al.* (2005)
Asian seabass (*Lates calcarifer*)	Δ6 Fad, Elovl5	Mohd-Yusof *et al.* (2010)
Bluefin tuna (*Thunnus thynnus*)	Δ6 Fad, Elovl5	Morais *et al.* (2011)
Rainbow trout (*Oncorhynchus mykiss*)	Δ6, Δ8 Fads	Seiliez *et al.* (2001); Zheng *et al.* (2004)
European seabass (*Dicentrarchus labrax*)	Δ6 Fad	González-Rovira *et al.* (2009); Santigosa *et al.* (2011)
Spotted-scat *(Scatophagus argus)*	Δ4 Fad	Xie *et al.* (2016)
Senegalese sole (*S. senegalensis*)	Δ4 Fad	Li *et al.* (2010); Morais *et al.* (2012)
Yellow croaker (*Larimichthys crocea*)	Elovl4, Elovl5	Li *et al.* (2017b)
Nibe croaker (*Nibea nmitsukurii*)	Elovl4	Kabeya *et al.* (2015)
Grouper (*E. coioides*)	Elovl4	Li *et al.* (2017a)

The long chain PUFAs (LC-PUFAs), including the docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA), play important roles in the growth performance, development and reproduction of fish and other vertebrates (Sargent, 1993; Sargent *et al.*, 1993; 1995; Sargent and Henderson, 1995; Sargent *et al.*, 1997). The roles of three PUFA's in fish are summarized into two categories: (i) to

maintain the structural and functional integrity of the cell membrane and (ii) to act as precursors of the highly biologically active paracrine hormones such as eicosanoids. The high dietary levels of DHA contribute to the neural development (structure and function of the cell membrane of fish); the high dietary levels of EPA and ARA are involved in the determination of eicosanoid actions (Sargent, 1993; Sargent *et al.*, 1993; 1995; Sargent and Henderson, 1995; Sargent *et al.*, 1997). In the mass production of marine fish fry for aquaculture, finding the appropriate composition of EPA, DHA and ARA in the body of each species of marine fish larvae is crucial for the establishment of culture technology, artificial food and EPA/DHA-enriched live foods (Watanabe *et al.*, 1978b; Watanabe *et al.*, 1978a; Watanabe *et al.*, 1978c; Imada *et al.*, 1979; Kitajima *et al.*, 1979; Watanabe *et al.*, 1982; Watanabe *et al.*, 1984a; Watanabe *et al.*, 1984b; Ogata *et al.*, 2004) (Table 2.5). Furthermore, the dietary PUFA composition also had an important effect on brood stocks' performance, including the fecundity, embryo development, hatchability and survival (Izquierdo *et al.*, 2001). Several studies on the PUFA composition in marine fish brood stocks and larval diets were carried out (Gibson, 1983; Gibson *et al.*, 1984; Fogerty *et al.*, 1986; Ako *et al.*, 1994b; Castell *et al.*, 1994; Sargent *et al.*, 1999a; Sargent *et al.*, 1999b; Bell and Sargent, 2003). It is reported that the wild tropical/subtropical marine fish contained ARA levels equivalent to or higher than those of EPA. By contrast, the ARA levels are lower than EPA in cold-water species (Gibson, 1983; Gibson *et al.*, 1984; Fogerty *et al.*, 1986; Ako *et al.*, 1994b; Castell *et al.*, 1994; Sargent *et al.*, 1999a; Sargent *et al.*, 1999b; Bell and Sargent, 2003). Within a similar proximate composition and n-3 HUFA level, the eggs and larvae performed better with higher ARA content in the diets in sea bass (Bell *et al.*, 1997; Bruce *et al.*, 1999), sea bream (Bessonart *et al.*, 1999) and Japanese flounder (Furuita *et al.*, 2000; Furuita *et al.*, 2003) and Atlantic cod (Zheng *et al.*, 1996). In nature, EPA and DHA in fish have been reported to come from phytoplankton and aquatic plants, which are consumed by herbivores and subsequently delivered into carnivores (Sargent and Whittle, 1981), while ARA origin is unclear. Johns *et al.* (1979) reported that red/brown macro-algae and microalgae might be ARA sources for marine fish. Indeed, the ARA content in red algae (*Gracilaria sp*.) in Vietnam ranges from 5.9 to 54.8% of total lipids (Imbs *et al.*, 2012). In conclusion, since siganids are herbivores, benthic macro algae are the main source of external PUFA of this group of fish (Suloma and Ogata, 2011). The information of PUFA and ratio of DHA/EPA in the body of fish larvae can be used as a

guideline for development of appropriate brood stock and larval diets, to ensure high egg and larval quality originating from sustainable hatchery production in tropical/subtropical areas (Watanabe *et al.*, 1978b; Watanabe *et al.*, 1978a; Watanabe *et al.*, 1978c; Imada *et al.*, 1979; Kitajima *et al.*, 1979; Watanabe *et al.*, 1982; Watanabe *et al.*, 1984a; Watanabe *et al.*, 1984b; Ogata *et al.*, 2004).

Table 2.5. PUFA composition of the lipid (mean % ± S.E.) of five tropical marine aquaculture fish (Ogata *et al.*, 2004; Nayak *et al.*, 2017). For *Siganus sp.* and *Caranx fulvoguttatus*: mean of 2 pooled samples from 10 fish); for *Lutjanus argentimaculatus:* mean of 3 fish.

LC-PUFAs	*S. guttatus*	*S. canaliculatus*	*S. rivulatus*	*L. argentimaculatus*	*C. fulvoguttatus*
ARA	0.6 ± 0.0	4.0 ± 0.8	2.0 ± 0.1	3.5 ± 0.9	-
EPA	1.1 ± 0.1	2.7 ± 0.9	0.4 ± 0.0	1.1 ± 0.0	-
DHA	5.7 ± 0.2	12.0 ± 1.9	5.0 ± 0.4	4.1 ± 1.0	-
Total (n-6)	18.6 ± 0.3	6.3 ± 0.9	-	8.2 ± 0.5	-
Total (n-3) HUFA	9.7 ± 0.4	18.4 ± 2.7	-	6.1 ± 0.0	-
ARA/EPA	0.6 ± 0.0	1.58 ± 0.20	0.2	3.3 ± 0.8	1.0
DHA/EPA	5.36 ± 0.10	4.76 ± 0.80	2.5	3.8 ± 0.8	5.8

2.6. Gut MC and roles of LAB and *B. subtilis* in fish gut

2.6.1. Gut MC of fish

Bacteria found in most parts of the fish body, such as gills, the skin and on inner surfaces, are usually harmless, symbiotic and reflect the MC from the surrounding environment. However, none of the bacteria found in those organs are as important as those of GI tract (Hansen and Olafsen, 1989). The fish gut MC are divided into two main groups: the allochthonous MC (contain bacteria which pass through the lumen with food) and the autochthonous MC (contain potentially fish gut colonizing bacteria) (Ringø and Birkbeck, 1999; Romero *et al.*, 2014). A normal gut MC is identified as a MC with colonies in most individuals' gut of a fish population or the ones which are present in various tissues within a fish body, including gut tissues, but cause no harm to the fish (Berg, 1996; Romero *et al.*, 2014). The gut MC is considered as an additional organ of the fish, which influences a wide variety of host's functions, such as the growth performance and development, digestion, immune response and disease resistance (Rawls *et al.,* 2004; Romero *et al.,* 2014). The gut MC composition indicated the interactions between host and the natural environment (O'Hara and Shanahan, 2006; Dhanasiri *et al.*, 2011; Dehler *et al.*, 2017). The gut MC can also

provide extra nutrients and extracellular enzymes, fatty acids and vitamins, which are limited or cannot be produced by the host themselves (Dhanasiri *et al.*, 2011), in return, the host offers a niche for some bacteria to benefit from their dietary life style. In general, it is important to understand the composition of the gut MC (Dhanasiri *et al.*, 2011; van Kessel *et al.*, 2011; Romero *et al.*, 2014). Previous studies showed that there are several factors influencing the gut MC diversity and composition, such as the host organism itself (Li *et al.,* 2014; Givens *et al.,* 2015), the stage in the life cycle (Giatsis *et al.*, 2014; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b; Zarkasi *et al.*, 2014; Zarkasi *et al.*, 2016), environmental factors (Hagi *et al.,* 2004; Navarrete *et al.,* 2009; Navarrete *et al.,* 2012; Romero *et al.,* 2014) and dietary life style (Navarrete *et al.*, 2013; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b). In fact, the fish gut MC has stronger interactions and is more influenced by the outside environment, than it is the case in other vertebrates, because of the permanent contact with water (Austin, 2006; Ringø *et al.*, 2010; Merrifield and Ringø, 2014). A gut MC of fish larvae could be detected shortly after the mouth opened, even before the first feeding happened, due to the drinking of water (Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b). At first feeding, the MC from the food is absorbed, attached and colonizing the gut of fish larvae (Austin, 2006; Benson *et al.*, 2010; Ringø *et al.*, 2010; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b). More importantly, due to the digestion process, only specific bacteria are retained, others are released into the external environment or digested by the fish enzymes. In addition, the majority of bacteria in the gut content are symbiotic species (in the sense of mutual benefit), compared to the free-living bacteria. In other words, the gut MC is not made up passively from the seeding communities (Nayak, 2010; Navarrete *et al.*, 2012; Abid *et al.*, 2013; Li *et al.*, 2014; Llewellyn *et al.*, 2014; Givens *et al.*, 2015). Since the genetic background and ontogeny are very difficult to be influenced, feeding appeared to be the most convenient way to manipulate the gut MC. Most bacteria in the gut MC are delivered through food consumed by the fish (Cahill, 1990; Ringø and Birkbeck, 1999; Romero and Navarrete, 2006; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b). The strong effects of feeding on the gut MC composition are also confirmed by Wu *et al.* (2012a); Wu *et al.* (2012b); Wu *et al.* (2013); and Hu *et al.* (2014). In aquaculture, the LAB and *Bacillus sp.,* are widely used to control diseases and enhance the nutrient intake by the fish (Verschuere *et al.*, 2000; Ringø *et al.*, 2010). Their ability to colonize successfully the fish gut content was investigated by Balcázar *et al.* (2007); Ringø *et al.* (2010); Dawood *et al.*

(2016) and Navarrete *et al.* (2013). Bioencapsulation of bacteria in rotifers or *Artemia* is the most convenient way to deliver LAB and *B. subtilis* to the gut of fish larvae (Verschuere *et al.,* 1999; Makridis *et al.,* 2000b; Vadstein *et al*., 2013). The number and composition of bacteria of live food are influenced by the cultivation method and live food MC affects the gut MC of fish larvae (Attramadal *et al.*, 2012a; Attramadal *et al.*, 2012b). The beneficial effects of probiotics in the gut of fish larvae are diverse, *e.g.* (i) digestive enzyme excretion, (ii) immune stimulation, (iii) production of antagonistic compounds and (iv) competitive exclusion (De Schryver *et al.,* 2012). Several studies reported that gut MC of fish larvae at first feeding was dominated by probiotics bioencapsulated in live food (Suzer et al. 2008; Vadstein et al., 2013). By contrast, in the later stage of larvae and juveniles, where the stomach barrier and gut MC have already been established, the presence of probiotics in the gut is dependent on the continuous addition of probiotics via feeding. Hence, the colonization success of intestinal epithelium by probiotics is still questioned (Makridis *et al.*, 2000; Makridis *et al.*, 2008; Bakke *et al.*, 2013; Vadstein *et al.*, 2013).

The gut MC of fish is investigated in many studies, via culture-dependent (bacterial cultured *ex vivo*) and culture-independent techniques (16S rRNA amplication). The 16S rRNA can be analyzed by DGGE, TGGE, T-RFLP, and pyrosequencing…). These studies have demonstrated the specificity of intestinal MC in fish (Amann, 1995; Amann *et al.*, 1995; Romero and Navarrete, 2006; Hovda *et al.*, 2007; Kim *et al.*, 2007; Navarrete *et al.*, 2009; Nayak, 2010; Wu *et al.*, 2010; Roeselers *et al.*, 2011; Green *et al.*, 2013; Wu *et al.*, 2013). However, the number of bacterial species identified in the fish gut is limited in these studies. For example, only 1% of bacteria are able to grow on culture media, and only predominant bacteria (>1% abundance) can be detected by DGGE or TGGE (Skrodenyte-Arbaciauskiene *et al.*, 2008; Merrifield *et al.*, 2009; Navarrete *et al.*, 2009; Navarrete *et al.*, 2012; Reveco *et al.*, 2014). In recent years, the technological development in advanced sequencing methods (for MC analysis, on amplified 16s rDNA sequences), for example, Roches' 454 and Illumina MiSeq, NextSeq 500, and HiSeq 2000 have resolved the limitations of the previous technologies (Romero and Navarrete, 2006; Hovda *et al.*, 2007; Navarrete *et al.*, 2009; van Kessel *et al.*, 2011; Geraylou *et al.*, 2013; Green *et al.*, 2013; Star *et al.*, 2013; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b; Zarkasi *et al.*, 2014; Zarkasi *et al.*, 2016). With these advanced molecular technologies, more

comprehensive results on diversity and composition of MC in the fish gut have been obtained (Austin, 2006; Kim *et al.*, 2007; Namba *et al.*, 2007; Lan and Love, 2012; Larsen *et al.*, 2013; Reveco *et al.*, 2014; Dehler *et al.*, 2017; He *et al.*, 2017). This is because readings of *e.g.* 100,000 sequences per biological sample provide enough information for the detection and enumeration of rare genera and/or species (DeSantis *et al.*, 2006; Liu *et al.*, 2007; Pruesse *et al.*, 2007; Wang *et al.*, 2007; Hamady and Knight, 2009; Romero *et al.*, 2014). The main objective of fish gut MC studies is to generate scientific knowledge on community structure and function eventually allowing for gut MC manipulation, in order to enhance the fish growth performance, health and immune response (Romero *et al.*, 2014).

2.6.2. LAB and *B. subtilis*

Lactic acid bacteria (LAB) are Gram-positive, non-mobile, non-sporulating and nutritionally fastidious bacteria, which convert nutrients (carbohydrates, amino acids, peptides, nucleic acid derivate and vitamins) into lactic acid as main product via fermentation metabolism (Stanier *et al.*, 1975; Lauzon *et al.*, 2014) (Table 2.6).

Table 2.6. Basic differences between groups of LAB and *B. subtilis,* according to Ringø and Gatesoupe (1998); Moszer *et al.* (2002); Sonenshein *et al.* (2002), Merrifield and Ringø, (2014).

No.	Genus name	Single cells	Cells arrangement	Fermentation
1	Streptococcus	cocci	pair, chain	homolactic
2	Leucococcus	cocci	pair, chain	heterolactic
3	Enterococcus	cocci	pair, chain	homolactic
4	Vagococcus	cocci, rods	pair, chain	homolactic
5	Lactobacillus	rods	pair, chain	homo/heterolactic
6	Carnobacterium	rods	pair, chain	heterolactic
7	Pediococcus	cocci	tetra, cluster	homolactic
8	Aerococcus	cocci	tetra, cluster	homolactic

The LAB adapt and grow under different environmental conditions and are often found in the GI tract of terrestrial and aquatic animals (Tannock and Savage, 1974; Tannock *et al.*, 1982; Finegold *et al.*, 1983; Tannock, 1983; 1990; Ringø *et al.*, 1995). LAB are basically considered as a favorable group of bacteria because of their antagonistic abilities against pathogens. They are commonly found as components in the gut MC of fish. The LAB included the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus.* These genera can be isolated by culture-dependent methods from the fish gut content (Lauzon *et al.*, 2014; Merrifield *et al.*, 2014;

Merrifield and Carnevali, 2014; Merrifield and Ringø, 2014). *B. subtilis* are endospore forming Gram-positive bacteria (Moszer *et al.*, 2002; Sonenshein *et al.*, 2002). *B. subtilis* and its spores could be isolated from the fish gut and are widely used in aquaculture due to their immunostimulatory properties in the GI immune system (Gatesoupe, 1999).

2.6.3. The roles of LAB and *B. subtilis* in fish gut

The presence of LAB in the GI tract of fish was reported by Ringø *et al.* (1995); Ringø and Vadstein (1998); Merrifield and Ringø (2014). Upon hatching, the fish gut is sterile. After that, the gut is colonized by a variety of MC coming from the food and the external environment (Campbell and Buswell, 1983; Muroga *et al.*, 1987; Munro *et al.*, 1994; Ringø and Strøm, 1994; Ringø *et al.*, 1996). The gut MC is defined as autochthonous (bacteria can colonize the host's epithelium/microvilli), or as allochthonous when they cannot (Ringø and Birkbeck, 1999; Merrifield *et al.*, 2014; Hoseinifar *et al.*, 2016). The larval gut MC composition is regulated by type of food and feeding, host physiology, MC in the water, stress and immunology (Ringø and Gatesoupe, 1998; Merrifield and Ringø, 2014). Rombout et al. (1984) described that the establishment of digestive enzymes influences the establishment of a healthy gut flora. Strøm and Olafsen (1990); Strøm and Ringø (1993) and Merrifield and Ringø (2014) reported that Gram-positive bacteria including LAB and *B. subtilis* could be delivered to the GI tract of larvae through feeding at their early life stage. However, the LAB and *B. subtilis* are seldom isolated from the gut of fish larvae in the wild. During the first few weeks after hatching, although the gut lengthens, twists and develops pouches for some specific functions, it is still very short compared to that of the adult fish. Based on the observation that LAB and *B. subtilis* are seldom isolated from the larval gut, various studies are performed on the possibility to help LAB and *B. subtilis* inhabit and colonize the GI tract of fish larvae and act as probiotics in the hatchery (Tannock *et al.*, 1982; Gatesoupe, 1989; 1991a; Kjørsvik *et al.*, 1991; Gildberg *et al.*, 1997; Ringø and Birkbeck, 1999; Gomez-Gil *et al.*, 2000; Olafsen, 2001; Gatesoupe, 2002; Hjelm *et al.*, 2004; Planas *et al.*, 2006; Bagheri *et al.*, 2008; Suzer *et al.*, 2008; Tovar-Ramírez *et al.*, 2010).

The LAB and *B. subtilis* are widely used in fish larval rearing as probiotics. Firstly, they are able to colonize the GI tract at early stage (Strøm and Olafsen, 1990; Strøm and Ringø, 1993; Merrifield and Ringø, 2014). Strøm and Ringø (1993) reported that upon

addition in the rearing water, LAB could constitute up to 70% of the total bacterial count in cod intestines. At first feeding, it is possible to enrich the rotifer *B. plicatilis* with *Lactobacillus* and *Carnobacterium* prior to feeding to the fish larvae (Gatesoupe, 1994). At weaning to compound diets or in salmonids (fed directly on compound diets), the LAB and *B. subtilis* could be introduced by feeding the larvae with food impregnated with probiotics. In this way, probiotics could persist in the larval gut to up to 3 days (Muroga *et al.*, 1987; Gildberg *et al.*, 1995; Gildberg *et al.*, 1997; Jöborn *et al.*, 1997). Secondly, the LAB and *B. subtilis* could adhere to the mucus of the GI epithelium. The adhesion activity is the first step of the colonization of a microorganism (AFRC, 1989; Fuller, 1991; Havenaar and Huis, 1992; Collins and Gibson, 1999; Schrezenmeir and de Vrese, 2001). The presence of LAB and *B. subtilis* enhance the growth and feeding performance, survival, gut MC, ammonia and urea excretion in Atlantic cod (*G. morhua*) (Strøm and Ringø, 1993), Nile tilapia (*O. niloticus*) (Lara-Flores *et al.*, 2003), gilthead sea bream (*S. aurata*) (Suzer *et al.*, 2008), Persian sturgeon (*Acipenser persicus*) (Askarian *et al.*, 2009; Faramarzi *et al.*, 2011; Hoseinifar *et al.*, 2016) and red sea bream (*P. major*) (Dawood *et al.*, 2016). Thirdly, LAB and *B. subtilis* presence in the gut mucosa provided antagonistic activity, by producing specific compounds such as lactic and other organic acids. These compounds inhibited the proliferation of other proteolytic bacteria, thus protecting the fish from pathogens (Shahani *et al.*, 1977; Hurst, 1981; Stoffels *et al.*, 1992; Merrifield *et al.*, 2014; Hoseinifar *et al.*, 2016). Fourthly, some LAB and *B. subtilis* produce bacteriocins, bactericidal or bacteriostatic peptides, which are antibacterial substances, *e.g. L. lactis* releases nisin, which inhibits the growth of *Aeromonas hydrophila*. *B. subtilis* also produces small molecular siderophores (<5 kDa) which have a wide spectrum of activity against bacterial pathogens in the fish intestine (Sugita *et al.*, 1996; Sugita *et al.*, 1998). Lastly, Balcázar *et al.* (2008) reported that *L. plantarum* and *L. fermentum* reduced the mucus adhesive capacity of *A. hydrophila* and *A. salmonicida* and *Yersinia ruckeri*. In conclusion, the use of probiotics should be considered in larviculture of fish, in order to improve health and quality of the fish larvae. However, Skjermo *et al.* (2015) reported that some probiotic bacteria, for example, *Microbacterium* (ID3-10), *Ruegeria* (RA4-1), *Pseudoalteromonas* (RA7-14) and *Vibrio* (RD5-30) are not predominant in the gut MC of fish, as they could not survive in the gut for a more than 4 days. Therefore, a constant input of probiotics

through feeding is highly recommended to provide a greater effectiveness for the control of infection caused by bacterial pathogens (Skjermo *et al.*, 2015).

In summary, the relative short time from initial feeding to oil globule exhaustion suggests that rabbit fish larvae will be more difficult to rear than either milkfish or seabass, as they would need very small live prey as starter food. Hence, a large-scale culture protocol for *P. similis* needs to be developed. After that, live food and probiotic feeding protocols using *P. similis* and *B. rotundiformis* as starter food will be tested from hatching to day 25. Also, the LC-PUFAs and gut MC compositions of wild and hatchery larvae will also be investigated. It was anticipated that this type of information on wild larvae would generate information to further optimise the larviculture of rabbit fish in the future.

CHAPTER 3

Growth performance of the very small rotifer *Proales similis* is more dependent on the proliferating bacterial community than the bigger rotifer *Brachionus rotundiformis*

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Abstract

P. similis and *B. rotundiformis* are commercially cultured rotifer species, which predominantly feed on microalgae. However, the importance of the bacterial community on growth performance of *P. similis* and *B. rotundiformis* culture is still unknown. In this study, the effect of limiting the bacterial growth and, as a second aim, the effect of the addition of a small amount of live or dead bacteria on the growth performance and MC of *P. similis* or *B. rotundiformis* culture was evaluated for a 10 day culture period. A rotifer culture with non-manipulated MC and fed autoclaved algae was used as the first control, and a culture started in autoclaved sea water and fed autoclaved algae was used as the second control. In order to test a food effect, probiotics and other bacteria present in the culture system were killed (inhibited to grow) through the addition of an antibiotic (AB) mixture and the rotifer culture performance was compared to that of a culture to which live probionts were added. In the presence of the live probiotic mixture, both rotifers species showed a better growth performance than those without the presence of probiotic mixture or those with the AB added. In addition, the growth performance of the rotifer *P. similis* is more dependent on proliferating bacterial community than the rotifer *B. rotundiformis*. The supplementation of these probiotic bacteria not only increased the production of the rotifers, but also had a regulating effect on the microbiota. The bacterial density was below detection limit in TCBS, MRS agar and MA in all treatments using AB during the culture period for both rotifer species.

Keywords: Rotifers, *Proales similis*; *Brachionus rotundiformis*; probiotics; growth performance; microbial community.

3.1. Introduction

Rotifera are one of the smallest metazoa of which over 2200 species have been described. Although *B. plicatilis* was first regarded as a pest in a pond, Japanese researchers soon realized that they could be used as a suitable live food organism for the early stage of marine fish. The successful use of rotifers in the commercial hatchery operations of the red sea bream (*P. major*) encouraged investigations in the development of mass culture techniques of rotifers (Fukusho and Iwamoto, 1981; Fukusho, 1989; Dhert *et al.*, 1995; Lavens and Sorgeloos, 1996; Hirata *et al.*, 1998). The *B. plicatilis* species complex is a group of euryhaline rotifers in the Brachionidae family. They are raised in the aquaculture industry as a food for most fish larvae. A simple classification is still used in aquaculture based on 3 different morphotypes, namely super small (SS-type), small (S-type), and large (L-type) rotifers (Dhert *et al.*, 1995; Hagiwara *et al.*, 1995; Lavens and Sorgeloos, 1996; Hagiwara *et al.*, 2001) although DNA-based characterization has been developed (Gómez and Carvalho, 2000; Papakostas *et al.*, 2006) and currently classified into 15 species (Mills *et al.*, 2016). In 2009, Wullur *et al*. reported that *P. similis*, with a body size of 83 ± 11 µm in length and 40 ± 6 µm in width, is a very small rotifer with a potential as first food for small mouth marine fish larvae. In aquaculture, the *P. similis* has recently reported to be one of the most suitable live food for the first feeding stage of marine species with small mouth/special oesophagus, including the seven-band grouper *E. septemfasciatus*, rusty angelfish *C. ferrugata*, humphead wrasse *C. undulatus* and Japanese eel *A. japonica* (Wullur *et al.*, 2009; 2011; Hirai *et al.*, 2012; Hirai *et al.*, 2013; Wullur *et al.*, 2013; Hagiwara *et al.*, 2014). Wullur *et al.* (2009) reported that *P. similis,* starting at 25 ind.mL^{-1}, can reach 250 ind.mL^{-1} at day 8 without aeration, and 2400 ind.mL^{-1} with aeration at day 11 at 25°C, 25 g.L^{-1} salinity. It was suggested that they could best be cultured with small microalgae such as *N. oculata* and *C. vulgaris*. In a batch culture, *P. similis* growth rate during the first 4 days of culture is usually lower than later on. This is a long lag-phase (Wullur *et al.*, 2009).

Bacteria are also known to be an important food for *Brachionus spp.* in natural conditions (Starkweather, 1980). Especially in the absence or low microalgae density, rotifers take up bacteria-size particles (Agasild and Nõges, 2005). *Pseudomonas* and *Acinetobacter* are common bacteria, which may constitute an important additional food

source for rotifers. Some *Pseudomonas* species, for instance, synthesize vitamin B_{12} which can be a limiting factor for rotifers under culture conditions (Yu *et al.*, 1988). The rotifer performance in bacteria-free conditions was worse than in a culture supplemented with a MC (Douillet, 1998; Rombaut *et al.*, 1999; Tinh *et al.*, 2006) indicating that bacteria are important to rotifers. Although bacteria can be considered as an alternative food for rotifers, they are considered to be a nuisance for a successful culture of marine fish larvae (bacteria associated with rotifers culture that might be harmful to the fish larvae (Gatesoupe *et al.*, 1989) *e.g. V. anguillarum* (Planas *et al.*, 2006), and several methods have been developed to limit the transfer of bacteria from rotifer cultures to larval tanks (Muroga and Yasunobu, 1987; Tanasomwang and Muroga, 1989; Munro *et al.*, 1993; Vadstein *et al.*, 1993; Munro *et al.*, 1994; Skjermo *et al.*, 1997; Verschuere *et al.*, 1997; Skjermo and Vadstein, 1999; Makridis *et al.*, 2000; Rombaut *et al.*, 2003; Qi *et al.*, 2009). LAB and *B. subtilis* were often added in the rotifer culture systems for the increasing the rotifer production. The addition of putative LAB and *B. subtilis* significantly increased the rotifer *B. plicatilis* population density and growth rate (Gatesoupe, 1991b; Harzevili *et al.*, 1998a; Harzevili *et al.*, 1998b; Hirata *et al.*, 1998; Douillet, 2000). In addition, Planas and Cunha (1999) used terrestrial-origin LAB (*L. casei, P. acidilactici and L. lactic*) to improve the *B. plicatilis* growth rate by 8 – 13 times than those obtained in the controls.

In the present study, the putative beneficial effect of a mixture of LAB and *Bacillus subtilis* on the growth performance of *P. similis* or *B. rotundiformis* was verified as well as their effect on the MC composition.

3.2. Materials and methods

Rotifers: *P. similis* (Okinawa strain, Japan) and *B. rotundiformis* (Bali strain, Indonesia) were obtained from the Lab. of Aquaculture Biology, Nagasaki University, Japan. The rotifers were maintained in 1 L Erlenmeyer flasks at 28 ± 1˚C, and were fed *N. oculata* (Reed Mariculture, USA) daily at the density of 10^6 cells.mL^{-1}.

Bacterial preparation: Five LAB strains were used, namely: *Lactobacillus plantarum* (strain R_{22}, isolated from the gut of *Decapterus korehu,* unpublished data)*, L. fermentum (strain* TC_{19}, isolated from fermented *P. monodon)*, *L. brevis (strain* NC_2, isolated from fermented beef*), Pediococcus acidilactici (strain* TC_5, isolated from

fermented *Penaeus monodon and P. pentosaceus (strain MC$_1$t$_2$*, isolated from fermented bamboo shoot, unpublished data*)* and a strain of *B. subtilis* isolated from shrimp carapax *(strain C$_{10}$)* (Bose, 2011). All were obtained from the Lab. of Food Technology, Hue Uni. of Agriculture and Forestry, Vietnam. The LAB were cultured on the de Man, Rogosa and Sharpe (MRS, pH = 8) agar (Oxoid, UK) and *B. subtilis* was cultured on marine agar (MA, pH = 8) (Merck, Germany). From the pure culture of each strain, a single colony was removed, placed directly into 5 mL of MRS broth (pH = 8, 20 g.L^{-1} NaCl) for the LAB or marine broth (pH = 8) for *B. subtilis* and incubated overnight at 37 ˚C (LAB) or 28°C (*B. subtilis*) on a horizontal shaker (Kuhner shaker, Switzerland; 140 rpm). After 24h, the bacteria suspension was centrifuged at 2200 x*g* (Sanyo Mistral 2000R, Japan) for 15 minutes. The pellet re-suspended in 0.85% sterile saline solution and the density adjusted to give an optical density of 1 at 600 nm. The Miles & Misra method provided viable colony counts (Miles *et al.*, 1938) and then 10-fold serial dilutions were made to have approximately 1 x 10^6 CFU.mL^{-1}. This was the bacteria density used for all the probiotic assays performed in this study.

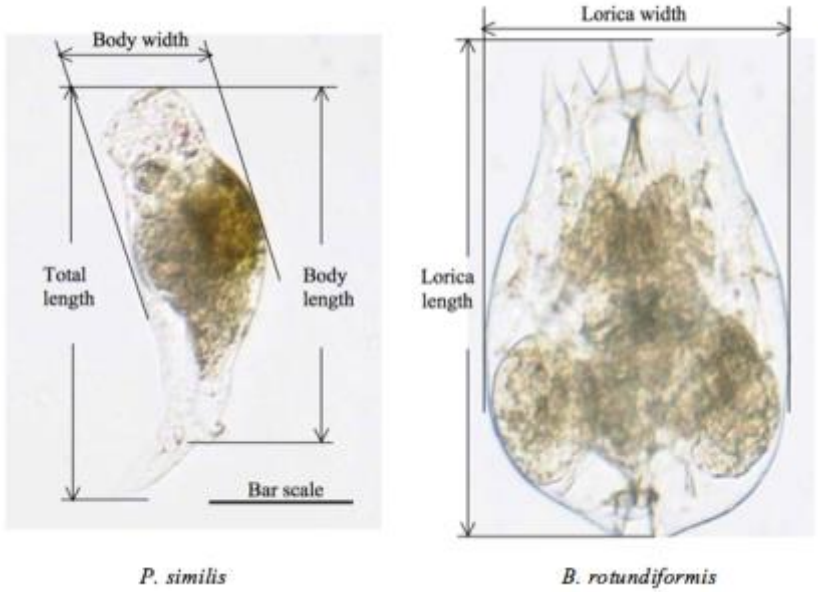


Figure 3.1. *P. similis* and SS-type *B. rotundiformis*. Bar = 50 µm (Wullur *et al.*, 2009).

3.2.3. Experimental design

3.2.3.1. Effect of ABs on the hatching success of *P. similis* and *B. rotundiformis*.

To monitor the effects of AB on the hatchability of *P. similis* and *B. rotundiformis*, 40 eggs of each rotifer species were collected and incubated individually in 22 g.L^{-1} autoclaved sea water at 28 ± 1°C with the addition of a mixture of four ABs; rifampicin

(Sigma-A83907), ampicillin (Sigma-A0166), trimethoprim (Sigma-T7883) and gentamycin (Sigma-G1264) (1:1:1:1). Three different concentrations (10, 50 or 100 $mg.L^{-1}$ of each AB) were evaluated. The treatment without addition of AB served as control. The number of hatched rotifers was determined 12 h after the start of the incubation.

3.2.3.2. Growth of LAB and *B. subtilis* in autoclaved rotifer culture medium

To test for the growth potential of probiotic bacteria in autoclaved rotifer culture medium, a mixture of the 6 probiotics was added (each strain at 10^6 $CFU.mL^{-1}.species^{-1}$ day^{-1}) to autoclaved water containing autoclaved algae at the same density as used during the rotifer tests. No rotifers were added. Water samples were collected every other day from day 2 for MC analysis through plating on MA (to check the total heterotrophic bacteria density), TCBS agar (Thiosulfate Citrate Bile Salts Sucrose, Merck, Germany) (to check for bacteria that can growth on TCBS) and MRS agar (to check the LAB density). The detection limit of the bacterial count was 20 $CFU.mL^{-1}$.

3.2.3.3. Effect of probiotics addition on the *P. similis* or *B. rotundiformis* performance

Rotifer and ABs preparation: All rotifers used to start experiments were newly hatched axenic rotifers. The axenic rotifers (*P. similis* and *B. rotundiformis*) were obtained by treating amictic eggs with glutaraldehyde according to the method from Tinh et al. (2006). Four ABs namely rifampicin (Sigma-A83907), ampicillin (Sigma-A0166), trimethoprim (Sigma-T7883) and gentamycin (Sigma-G1264) were prepared and added daily to the culture vials directly (each at 10 $mg.L^{-1}$ in the vials).

Experimental design for the effect of probiotics addition on the performance of rotifers: Two different groups of experiments were run in a *P. similis* culture and in a *B. rotundiformis* culture (Table 3.1). Both groups of experiments were run simultaneous, originating from the same batch of rotifers. In the first group, a culture of *P. similis* or *B. rotundiformis* with non-manipulated MC served as the control (1 P Cont and 1 B Cont, respectively). In treatment 1 P AB and 1 B AB, a mixture of four AB (rifampicin, ampicillin, trimethoprim and gentamycin, each at 10 $mg.L^{-1}$) was added to the culture system. In the second group, a culture of *P. similis* or *B. rotundiformis* started in autoclaved water and fed autoclaved algae served as the control (2 P Cont and 2 B Cont, respectively). A mixture of *L. plantarum, L. fermentum, L. brevis, P.*

acidilactici, P. pentosaceus and *B. subtilis* (10^6 CFU.mL^{-1} each, totally 6 x 10^6 CFU.mL^{-1}) and algae (10^6 cells.mL^{-1} day^{-1}) were added daily to the *P. similis* or *B. rotundiformis* cultures. In order to investigate whether the effects of the mixture of LAB and *B. subtilis* on the rotifer growth performance are nutritional or probiotic effects, the bacteria were added alive (treatment 2 P Prob and 2 B Prob) or added alive with AB present in the cultures (to limit proliferation of probionts and other bacteria) (Treatment 2 P Prob+AB and 2 B Prob+AB) (Table 1). All treatments were performed in 100 mL glass bottles containing 80 mL of seawater at a salinity of 22 g.L^{-1}, with four replicates per treatment and kept in darkness for 10 days. Water temperature was 28 ± 1°C. Water was not exchanged and no aeration was supplied during the experiment. Rotifers and water samples were collected every other day from day 2 onwards to determine the rotifer and microbial densities through plating on MA, TCBS and MRS agar. The detection limit of bacteria count was 20 CFU.mL^{-1}. Population growth rate of *P. similis* was compared to that of *B. rotundiformis* cultured in similar conditions. The growth rates (r) were calculated using the following equation (Krebs, 1985):

$$r = (lnNt - lnNo) / t$$

(ln = natural logarithm, Nt = population after time t (days) and No = initial population)

Table 3.1. Experimental design to test the effects of culture conditions on *P. similis* or *B. rotundiformis* performance and MC

Treatment group	*P. similis* culture	Treatment description	Aim	*B. rotundiformis* culture
Group 1	1 P Cont	Non-manipulated MC and non-autoclaved live algae		1 B Cont
	1 P AB	1 P Cont or 1 B Cont + mixture of four ABs	To test the effect of limiting bacterial growth	1 B AB
Group 2	2 P Cont	Started in autoclaved water and fed autoclaved algae		2 B Cont
	2 P Prob	2 P Cont or 2 B Cont + mixture of 6 species of tentative probiotic bacteria	To test the probiotic effect of some tentative probiotic bacteria	2 B Prob
	2 P Prob+AB	2 P Cont or 2 B Cont + mixture of 6 species of killed (inhibited to growth) tentative probiotic bacteria	To test the feeding effect of the tentative probiotic bacteria	2 B Prob+AB

3.2.3.4. Denaturing Gradient Gel Electrophoresis (DGGE)

DNA extraction and PCR amplification: From each replicate, 1 mL of sample (rotifers included) was collected at day 0, 6 and 10. The samples were freeze dried and stored at − 20 °C until further processing. Total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, The Netherlands) according to the manufacturer's instructions. PCR amplification targeting the V3 region of the 16S rRNA gene was conducted as described by Boon *et al.* (2002) using bacterial primer 338f (5'-ACTCCTACGGGAGGCAGCAG-3') with a 40-base GC clamp attached to its 5' end and universal primer 518r (5'-ATTACCGCGGCTGCTGG-3'). The PCR protocol consisted of 95 °C for 5 min, followed by 32 cycles with 95 °C 30 s, 55 °C 30 s, and 72 °C 60 s, and finally an elongation step at 72 °C for 10 min.

Denaturing gradient gel electrophoresis (DGGE): The DGGE was performed using a Bio-Rad D Gene system (Bio-Rad, USA) (Boon *et al.*, 2002). The denaturing gradient of the gel ranged between 45% and 60%. Electrophoresis was performed at a constant voltage of 38 V at 60 °C for 16 h. The DGGE gels were stained for 20 minutes in a 1% gel-red solution and the bands were visualized using a UV transilluminator (Bio-Rad).

Band sequencing: DGGE bands were cut out from the gel and incubated overnight at 4°C in 30 µL of H_2O. An aliquot (2 µL) was used in a PCR with the same primer set and primer protocol as used for DGGE, to reamplify the fragment. Aliquots (2 µL) of the products from the re-PCRs were cloned into a TA vector using the pGEM-T Easy Vector System I cloning kit (Promega) and then chemically transformed into the *E. coli* Top 10 (Invitrogen). The PCR cloning analysis of the *E. coli* colonies was conducted using the primers T7 (5'-TAATACGACTCACTATAGGG-3') and 518R. The transformed *E. coli* colonies were plated overnight and the DNA was extracted using the Wizard® *Plus* SV Minipreps DNA Purification Systems (Promega). Aliquots (10 µL**)** were used for the sequencing reactions with T7 and SP6 (5'-ATTTAGGTGACACTATAG-3') primers at LGC Genomics GmbH, Germany. Nucleotide sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (ABI). Sequences were analyzed on an ABI 3100 automatic sequencer. Generally, only a single strand of the DNA fragments were sequenced. This proved to be sufficient for the taxonomic identification of the cloned 16S rRNA gene fragments using the BLAST search function within the NCBI database.

3.2.4. Experimental design to test the effect of probiotics' addition on the performance of rotifers in large-scale culture

All treatments were performed in 400 L fiberglass cylindro-conical tanks containing 200 L of sea water at a salinity of 22 $g.L^{-1}$ and kept in darkness (four replicates per treatment). The experiment ran for 8 days. Water temperature was 28 ± 1°C. During the experiment, the seawater was not exchanged. Aeration was supplied by an air stone placed at the bottom center of each tank. Firstly, a culture of *P. similis* or *B. rotundiformis* started in filtered water (0.2 µm; Sartorius, Germany) and fed autoclaved algae with non-manipulated MC served as the control (control 1 and 2, respectively). Secondly, a culture of *P. similis* or *B. rotundiformis* cultured in filtered water (0.2 µm, Sartorius, Germany) and fed autoclaved algae (10^6 cells.mL^{-1}.day^{-1}) with the addition of a mixture of *L. plantarum, L. fermentum, L. brevis, P. acidilactici, P. pentosaceus* and *B. subtilis* (10^6 CFU.mL^{-1}.species^1 final density, totally 6 x 10^6 CFU.mL^{-1}) and algae paste (10^6 cells mL^{-1}.day^{-1}) (treatment 1 and 2, respectively). Rotifers were collected every other day from day 2 onwards to determine the rotifer densities. Population growth rate of *P. similis* was compared to those from *B. rotundiformis* cultured in similar conditions. The population growth rates (r) were calculated using the equation (Krebs, 1985) and the exponential population growth rates (r-max) were calculated according to Kostopoulou and Vadstein (2007).

3.2.5. Data analysis

Significant differences between means of continuous variables (rotifer population densities) in different treatments of each group were analyzed using one-way repeated measures of variance (rmANOVA). The significant differences in hatching success were compared by Pearson Chi-square test. The significant differences in rotifer densities (at each sampling point), growth rates and number of bacteria between treatments were compared by one-way ANOVA, followed by the Tukey-Kramer tests for multiple comparisons. Student t-tests were used where applicable. Probability level of $p<0.05$ was considered significant. All tests were performed using the SPSS program 22.0. The DGGE patterns were processed by Bionumerics software 5.0 (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed as described by Bakke *et al.* (2013). The band position tolerance limit to consider bands as being identical was set at 1%.

3.3. Results

3.3.1. Effect of ABs on hatching success of *P. similis* and *B. rotundiformis*

The toxicity of AB was evaluated based on the hatching success of *P. similis* and *B. rotundiformis*. The AB did not affect the hatchability of the eggs of *P. similis* and *B. rotundiformis* as there were no significant differences in hatching success between the control treatment (no AB added) and all AB treatments. The hatching success was 95% for *P. similis* eggs and from 95 to 100% for *B. rotundiformis* eggs (Pearson Chi-square test, $p>0.05$, $n = 40$).

3.3.2. Growth of LAB and *B. subtilis* in autoclaved rotifer culture medium

During the 10-day period, TCBS counts were always below detection limit. The number of bacteria on MRS agar and MA increased from $5.7 \pm 1.0 \times 10^7$ and $5.9 \pm 1.7 \times 10^8$ to $3.1 \pm 0.3 \times 10^8$ and $7.2 \pm 0.7 \times 10^9$ CFU.mL^{-1}, respectively.

3.3.3. Effect of probiotics addition on the population densities and growth rates of *P. similis* or *B. rotundiformis*

Overall, the population densities were significantly different among treatments during the experiment, except at the starting point (day 0) (Figure 3.2, rmANOVA, $p<0.001$). At each sampling time point, the population densities of *P. similis* and *B. rotundiformis* were significantly different among treatments, except at the starting point (day 0) (Figure 3.2, one-way ANOVA, $p<0.001$).

In group 1 experiments, except day 2, the population densities of *P. similis* (Figure 3.2A) and *B. rotundiformis* (Figure 3.2C) with the addition of a mixture of ANs (treatment 1 P AB: 75 ind.mL^{-1}; treatment 1 B AB: 74 ind.mL^{-1} at day 10) were always significantly lower (56 – 228%) than the controls (without AB addition) (treatment 1 P Cont: 243 ind.mL^{-1}, treatment 1 B Cont: 117 ind.mL^{-1} at day 10) (Tukey-Kramer, $p<0.05$).

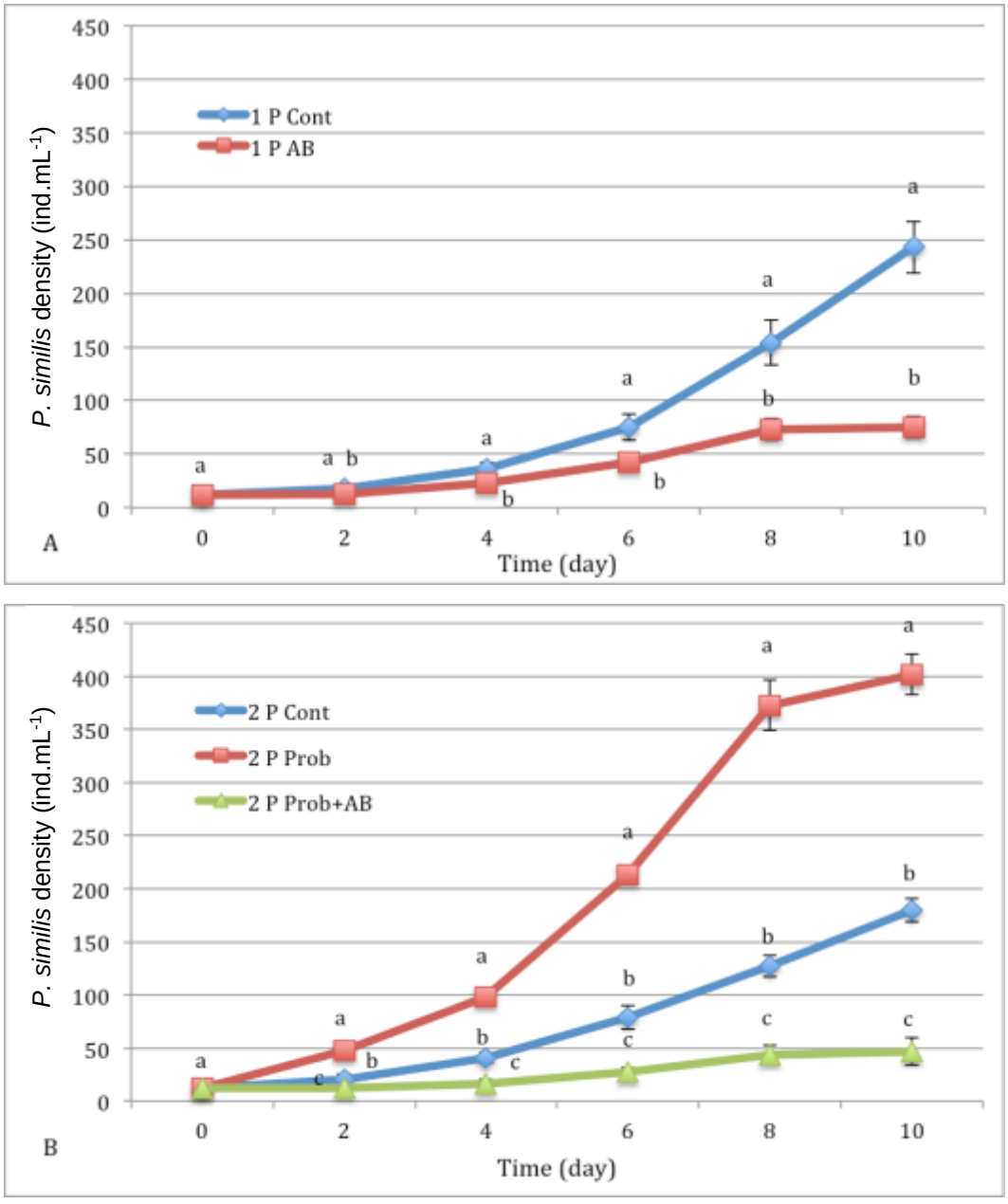
In group 2 experiments, the population densities of *P. similis* with the addition of the live mixture of bacteria (treatment 2 P Prob: 402 ind.mL^{-1} at day 10) was significantly higher than those in any other treatments from day 2 to day 10 (Tukey-Kramer, $p<0.05$). *P. similis* culture started in autoclaved water and fed autoclaved algae (2 P

Cont: 129 ind.mL^{-1} at day 10) showed a lower growth performance as compared to those fed algae and living probiotics (Tukey-Kramer, $p<0.05$), but showed higher population densities as compared to those fed probionts in the presence of AB (treatment 2 P Prob+AB) (Figure 3.2B). The same trend can be seen in *B. rotundiformis* cultures, where significant higher rotifer densities were observed in the treatment with putative probiotic bacteria addition (treatment 2 B Prob: 123 ind.mL^{-1} at day 10). The population densities of *B. rotundiformis* in the treatment using autoclaved water and algae with addition of the mixture of probionts in the presence of AB (treatment 2 B Prob+AB: 61 ind.mL^{-1} at day 10) was similar to the culture started in autoclaved water and fed autoclaved algae (2 B Cont: 66 ind.mL^{-1} at day 10) (Tukey-Kramer, $p>0.05$) (Figure 3.2D).

On day 10, the addition of the mixture of killed probionts resulted in a negative effect to *P. similis* performance compared to the treatment with the addition of a mixture of ABs, without addition of probionts (treatment 2 P Prob+AB: 47 ind.mL^{-1}, compared to the treatment 1 P AB: 75 ind.mL^{-1}) (Tukey-Kramer, $p<0.05$). The population density of *P. similis* with the addition of the live mixture of bacteria (402 ind.mL^{-1}) was 755% higher than those fed probionts in the presence of AB (47 ind.mL^{-1}) (Figure 3.2B). On the other hand, the population densities of *B. rotundiformis* with the addition of the live mixture of bacteria (123 ind.mL^{-1}) were only 101% higher than those fed probionts in the presence of AB (61 ind.mL^{-1}) (Table 3.2D) (Tukey-Kramer, $p<0.05$).

From day 0 to day 10, the population growth rates of *P. similis* and *B. rotundiformis* were significantly different among treatments (Table 3.2, one-way ANOVA, $p<0.001$). The growth rate of *P. similis* with the addition of the live mixture of bacteria (treatment 2 P Prob: 0.479 day^{-1}) was significantly higher (16 – 118%) than any other treatment from both rotifer cultures (Table 3.2) (Tukey-Kramer, $p<0.05$). On the other hand, there were no significant differences in the *B. rotundiformis* growth rates where putative probiotic bacteria were or were not added (treatment 2 B Prob: 0.412 day^{-1}; 1 B Cont: 0.407 day^{-1}) (Tukey-Kramer, $p>0.05$). In addition, the growth rate of *B. rotundiformis* with the addition of the live mixture of bacteria (treatment 2 B Prob: 0.412 day^{-1}) was significantly higher than (20%) those from the treatment fed probionts in the presence of AB (treatment 2 B Prob+AB: 0.342 day^{-1}) (Tukey-Kramer, $p<0.05$).
Overall, the rotifer cultures with addition of a mixture of ABs resulted in lower population densities and growth rates of the rotifers than the treatments without

addition of the ABs. Secondly, the rotifer cultures in which live probiotics were added led to a higher population densities and growth rates than treatments in which probiotics were used in the presence of AB, and treatments started in autoclaved water and fed autoclaved algae, but without probiotics addition. Finally, the growth performance of the rotifer *P. similis* appears to be more dependent on the proliferating bacterial community than the rotifer *B. rotundiformis.*



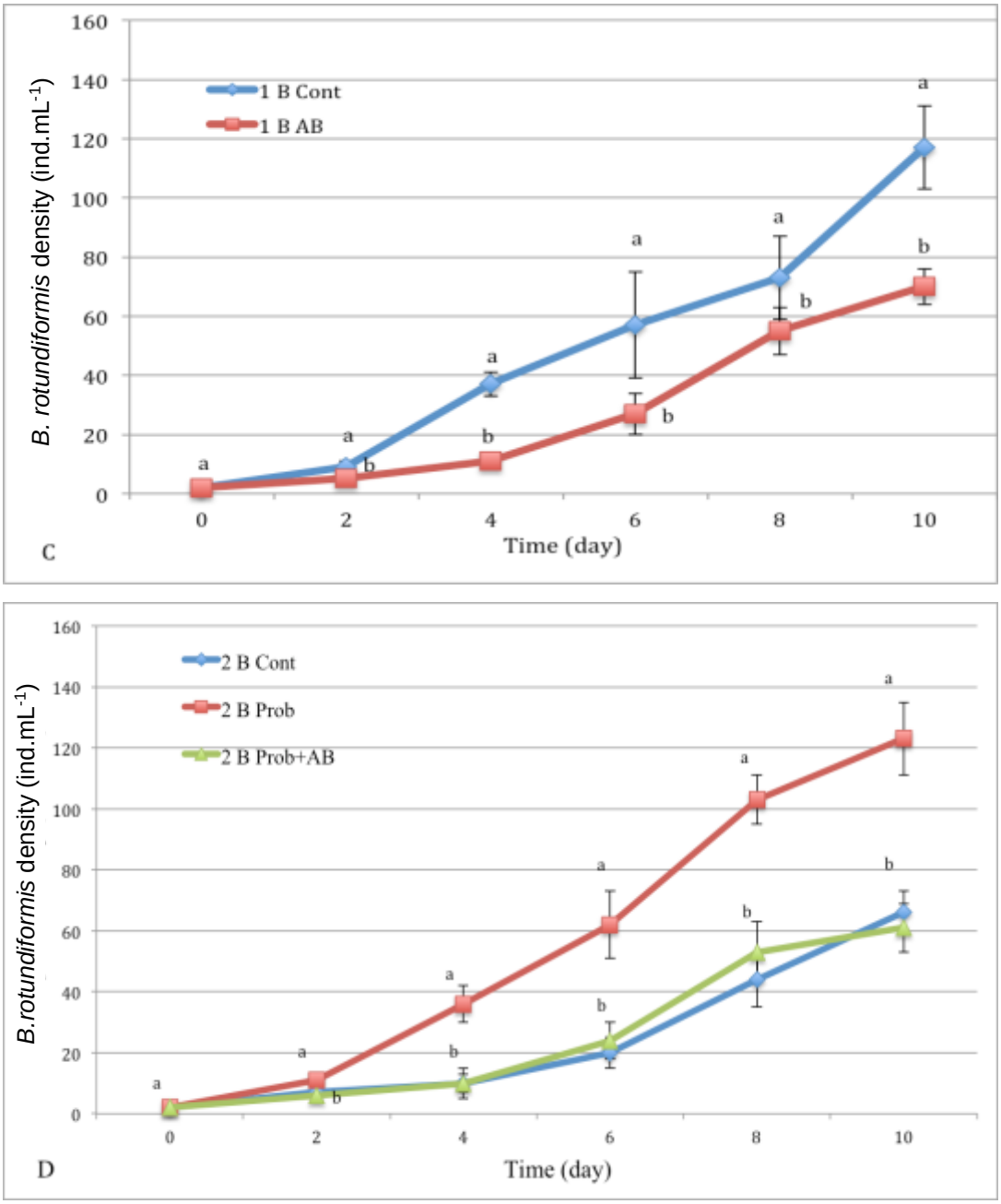


Figure 3.2. Growth performance of *P. similis* (A, B); *B. rotundiformis* (C, D) under 5 different culture conditions (mean ± SD, n = 4). 1 P Cont and 1 B Cont: non-manipulated MC and unautoclaved algae; Treatment 1 P AB and 1 B AB: non-manipulated MC and unautoclaved algae + mixture of four AB; 2 P Cont and 2 B Cont: started in autoclaved water and fed autoclaved algae; Treatment 2 P Prob and 2 B Prob: started in autoclaved water and fed autoclaved algae + mixture of six species of tentative probiotic bacteria; Treatment 2 P Prob+AB and 2 B: Prob+AB started in autoclaved water, fed autoclaved algae + mixture of six species of AB killed tentative probiotic bacteria. Significant differences between means in continuous variables (rotifer population densities) between treatments of each group during experiments were analyzed using one-way repeated measures of variance (rmANOVA). Different letters indicate significant differences in rotifer population densities among treatments at each sampling point (one-way ANOVA, Tukey-Kramer tests for multiple comparisons).

Table 3.2. *P. similis* and *B. rotundiformis* growth rates (day^{-1}) in 10 days (from day 0 to 10) in all treatments (mean ± S.D., n=4)

Cultures	Treatments*	Growth rate
P. similis	1 P Cont	0.354 ± 0.015^{d}
	1 P AB	0.283 ± 0.019^{b}
	2 P Cont	0.308 ± 0.023^{bc}
	2 P Prob	0.479 ± 0.002^{f}
	2 P Prob+AB	0.220 ± 0.030^{a}
B. rotundiformis	1 B Cont	0.407 ± 0.005^{e}
	1 B AB	0.356 ± 0.007^{d}
	2 B Cont	0.349 ± 0.003^{d}
	2 B Prob	0.412 ± 0.005^{e}
	2 B Prob+AB	0.342 ± 0.012^{cd}

(*) 1 P Cont and 1 B Cont: non-manipulated MC and unautoclaved algae; Treatment 1 P AB and 1 B AB: non-manipulated MC and unautoclaved algae + mixture of four AB; 2 P Cont and 2 B Cont: started in autoclaved water and fed autoclaved algae; Treatment 2 P Prob and 2 B Prob: started in autoclaved water and fed autoclaved algae + mixture of six species of tentative probiotic bacteria; Treatment 2 P Prob+AB and 2 B: Prob+AB started in autoclaved water, fed autoclaved algae + mixture of six species of AB killed tentative probiotic bacteria. Different letters from each column indicates significant differences in growth rates at day 10 among treatments across species (one-way ANOVA, Tukey-Kramer post-tests for multiple comparisons).

3.3.4. Bacteria counts

The MC in *P. similis* and *B. rotundiformis* cultures were assessed every 2 days by counting the number of colonies on TCBS agar, MRS agar and MA. Overall, the bacterial counts were significantly different among treatments during the experiment (rmANOVA, $p < 0.001$). At each sampling time point (day 2 and day 10), the bacteria counts were significantly different among treatments (one-way ANOVA, $p<0.001$) (Table 3.3). The number of bacteria on TCBS agar increased with time. In group 1, at day 10, the TCBS counts in the 1 B Cont (1.2×10^4 $CFU.mL^{-1}$) were significantly higher than in the 1 P Cont (4.0×10^3 $CFU.mL^{-1}$). This was also the case in group 2. At day 10, the TCBS counts in the 2 B Cont (3.1×10^4 $CFU.mL^{-1}$) were significantly higher than in the treatment 2 P Prob and 2 B Prob (Tukey-Kramer, $p<0.05$). The TCBS counts of treatment 1 P AB, 1 B AB, 2 P Prob+AB and 2 B Prob+AB was under detection limit (Table 3.3A). The number of bacteria on MRS agar in group 1 increased in all treatments without AB addition during the culture period. In group 2, the bacteria density in both cultures which included live probiotic addition was significantly higher than in any other treatments, and the bacteria density in *P. similis* cultures was

significantly lower than those in *B. rotundiformis* culture, at both day 2 and day 10 (4.2 x 10^4 CFU.mL^{-1} at the treatment 2 P Prob compared to 1.4 x 10^5 CFU.mL^{-1} at the treatment 2 B Prob at day 2, and 8.0 x 10^4 CFU.mL^{-1} at the treatment 2 P Prob compared 4.3 x 10^5 CFU.mL^{-1} in treatment 2 B Prob at day 10) (Tukey-Kramer, $p<0.05$). A significant difference was observed in the treatments without live probiotic addition (5.3 x 10^2 CFU.mL^{-1} at the 2 P Cont and 1.3 x 10^3 CFU.mL^{-1} at the 2 B Cont) (Tukey-Kramer, $p<0.05$) (Table 3.3B). In all growth media, the bacteria colonies were below detection limit in treatments with AB addition, while the number of bacteria increased in function of culture time in the other treatments. It seems that with or without addition of probiotics, the final bacterial density is around 10^8 CFU.mL^{-1} at day 10 in all treatments without the AB addition (Table 3.3C). Most importantly, within the same conditions, the bacterial densities in *P. similis* cultures were always higher than those in *B. rotundiformis* cultures at day 10 (3 – 6x on TCBS, 2 – 9x on MRS, 5 – 23x on MA) (Table 3.3).

3.3.5. Denaturing Gradient Gel Electrophoresis (DGGE)

The DGGE profiles obtained from the MC in all controls and treatments are shown in Figure 3.3. At day 6 and 10, the bands from the *L. plantarum* (band M1), *B. subtilis* (band M2), *L. brevis* (band M3), *P. acidilactici* (band M4), *P. pentosaceus* (band M4) *and L. fermentum* (band M5) were dominant in the treatments started in autoclaved water, fed autoclaved algae with the addition of the mixture of six species of tentative live or killed probiotic bacteria (columns 4, 5, 9, 10, 14, 15, 19 and 20) (the *P. acidilactici* and *P. pentosaceus* yield the same band on DGGE gel). In *P. similis* culture, two bands from *Vibrio sp.* (A1 and B1) appeared at day 6 of the treatments started in autoclaved water and fed autoclaved algae (column 3). At day 6 and 10, bands from *Vibrio sp.* (band A2 and B2) were also found in the same treatments. In *B. rotundiformis* culture, the bands from *Vibrio sp.* (band E1 and F1) were dominant at day 6 (column 13) in the treatments started in autoclaved water and fed autoclaved algae, whereas no *Bacillus sp.* or *Lactobacillus sp.* was visible at this point of the same treatment. However, the intensities of the *Vibrio sp.* bands (band E2 and F2) were reduced, while the bands from *Bacillus sp.* (band C) and *Lactobacillus sp.* (band D) were visible at day 10 (column 18).

Table 3.3A. Bacteria density (detected on TCBS agar) in all treatments* (mean ± S.D., CFU.mL^{-1}, n=4)

Group	Treatment	Days of culture				
		2	4	6	8	10
1	1 P Cont	$7.5 \pm 4.1 \times 10^{1a}$	$2.0 \pm 0.9 \times 10^{2}$	$1.3 \pm 0.8 \times 10^{3}$	$2.4 \pm 1.5 \times 10^{3}$	$4.0 \pm 2.1 \times 10^{3a}$
	1 B Cont	$4.8 \pm 1.5 \times 10^{2b}$	$8.5 \pm 4.1 \times 10^{2}$	$3.9 \pm 1.7 \times 10^{3}$	$7.2 \pm 1.8 \times 10^{3}$	$1.2 \pm 0.5 \times 10^{4b}$
2	2 P Cont	$2.5 \pm 1.0 \times 10^{1ac}$	$1.3 \pm 0.5 \times 10^{2}$	$1.1 \pm 0.7 \times 10^{3}$	$2.9 \pm 0.8 \times 10^{3}$	$5.9 \pm 2.0 \times 10^{3a}$
	2 P Prob	$1.5 \pm 0.6 \times 10^{1c}$	$3.0 \pm 2.0 \times 10^{1}$	$2.1 \pm 1.4 \times 10^{2}$	$5.5 \pm 3.0 \times 10^{2}$	$6.2 \pm 1.6 \times 10^{2c}$
	2 B Cont	$7.0 \pm 3.8 \times 10^{1d}$	$3.5 \pm 1.9 \times 10^{2}$	$3.0 \pm 1.8 \times 10^{3}$	$5.2 \pm 0.7 \times 10^{3}$	$3.1 \pm 1.3 \times 10^{4d}$
	2 B Prob	$4.0 \pm 2.8 \times 10^{1ac}$	$7.0 \pm 3.2 \times 10^{2}$	$1.6 \pm 0.2 \times 10^{3}$	$2.6 \pm 1.1 \times 10^{3}$	$6.8 \pm 1.7 \times 10^{3ab}$

Table 3.3B. Bacteria density (detected on MRS agar) in all treatments* (mean ± S.D., CFU.mL^{-1}, n=4)

Group	Treatment	Days of culture				
		2	4	6	8	10
1	1 P Cont	$0.5 \pm 0.2 \times 10^{1a}$	$1.5 \pm 0.6 \times 10^{1}$	$4.0 \pm 1.6 \times 10^{1}$	$2.8 \pm 1.0 \times 10^{2}$	$4.0 \pm 1.7 \times 10^{2a}$
	2 B Cont	$2.0 \pm 1.0 \times 10^{1b}$	$3.3 \pm 0.1 \times 10^{1}$	$8.8 \pm 2.2 \times 10^{2}$	$2.4 \pm 1.0 \times 10^{3}$	$3.7 \pm 1.1 \times 10^{3b}$
2	2 P Cont	$3.0 \pm 1.2 \times 10^{1b}$	$8.0 \pm 2.8 \times 10^{1}$	$2.3 \pm 1.0 \times 10^{2}$	$2.5 \pm 1.7 \times 10^{2}$	$5.3 \pm 2.2 \times 10^{2a}$
	2 P Prob	$4.2 \pm 1.8 \times 10^{4d}$	$4.6 \pm 0.8 \times 10^{4}$	$5.4 \pm 1.5 \times 10^{4}$	$6.8 \pm 2.5 \times 10^{4}$	$8.0 \pm 2.8 \times 10^{4d}$
	2 B Cont	$6.0 \pm 2.8 \times 10^{1b}$	$2.5 \pm 1.7 \times 10^{2}$	$5.8 \pm 1.7 \times 10^{2}$	$1.1 \pm 0.3 \times 10^{3}$	$1.3 \pm 0.4 \times 10^{3c}$
	2 B Prob	$1.4 \pm 0.4 \times 10^{5e}$	$1.7 \pm 0.3 \times 10^{5}$	$3.8 \pm 1.0 \times 10^{5}$	$4.0 \pm 0.9 \times 10^{5}$	$4.3 \pm 1.8 \times 10^{5e}$

Table 3.3C. Bacteria density (detected on MA) in all treatments* (mean ± S.D., CFU.mL^{-1}, n=4)

Group	Treatment	Days of culture				
		2	4	6	8	10
1	1 P Cont	$2.4 \pm 1.3 \times 10^{6abc}$	$3.2 \pm 1.0 \times 10^{6}$	$8.5 \pm 2.3 \times 10^{6}$	$2.0 \pm 0.9 \times 10^{7}$	$2.5 \pm 1.1 \times 10^{8a}$
	1 B Cont	$9.0 \pm 6.4 \times 10^{6a}$	$3.7 \pm 3.4 \times 10^{8}$	$4.4 \pm 3.2 \times 10^{8}$	$6.7 \pm 1.2 \times 10^{8}$	$1.9 \pm 1.2 \times 10^{9bc}$
2	2 P Cont	$6.5 \pm 2.0 \times 10^{5b}$	$4.0 \pm 2.0 \times 10^{6}$	$7.1 \pm 3.5 \times 10^{7}$	$8.9 \pm 3.7 \times 10^{7}$	$7.4 \pm 2.7 \times 10^{8ac}$
	2 P Prob	$4.9 \pm 2.4 \times 10^{6a}$	$7.5 \pm 2.3 \times 10^{6}$	$2.3 \pm 1.5 \times 10^{7}$	$5.4 \pm 4.4 \times 10^{7}$	$3.4 \pm 2.3 \times 10^{8ad}$
	2 B Cont	$1.1 \pm 0.5 \times 10^{5bc}$	$1.1 \pm 0.3 \times 10^{6}$	$2.5 \pm 3.5 \times 10^{8}$	$2.0 \pm 1.9 \times 10^{8}$	$4.0 \pm 1.3 \times 10^{9b}$
	2 B Prob	$3.8 \pm 4.1 \times 10^{6ac}$	$2.3 \pm 3.4 \times 10^{7}$	$3.9 \pm 2.4 \times 10^{7}$	$5.1 \pm 1.3 \times 10^{8}$	$8.0 \pm 1.3 \times 10^{8cd}$

1 P Cont & 1 B Cont: non-manipulated MC & unautoclaved algae; 1 P AB & 1 B AB: non-manipulated MC & unautoclaved algae + mixture of four AB; 2 P Cont & 2 B Cont: started in autoclaved water & fed autoclaved algae; 2 P Prob & 2 B Prob: started in autoclaved water, fed autoclaved algae & mixture of six species of tentative probiotic bacteria; 2 P Prob+AB & 2 B Prob+AB: started in autoclaved water, fed autoclaved algae & mixture of six species of AB killed tentative probiotic bacteria. Different letters indicate significant differences in bacterial densities among treatments on the same sampling point (one-way ANOVA, Tukey-Kramer tests for multiple comparisons). All bacterial densities measured in AB treatments (not shown in the table) were below detectable levels.

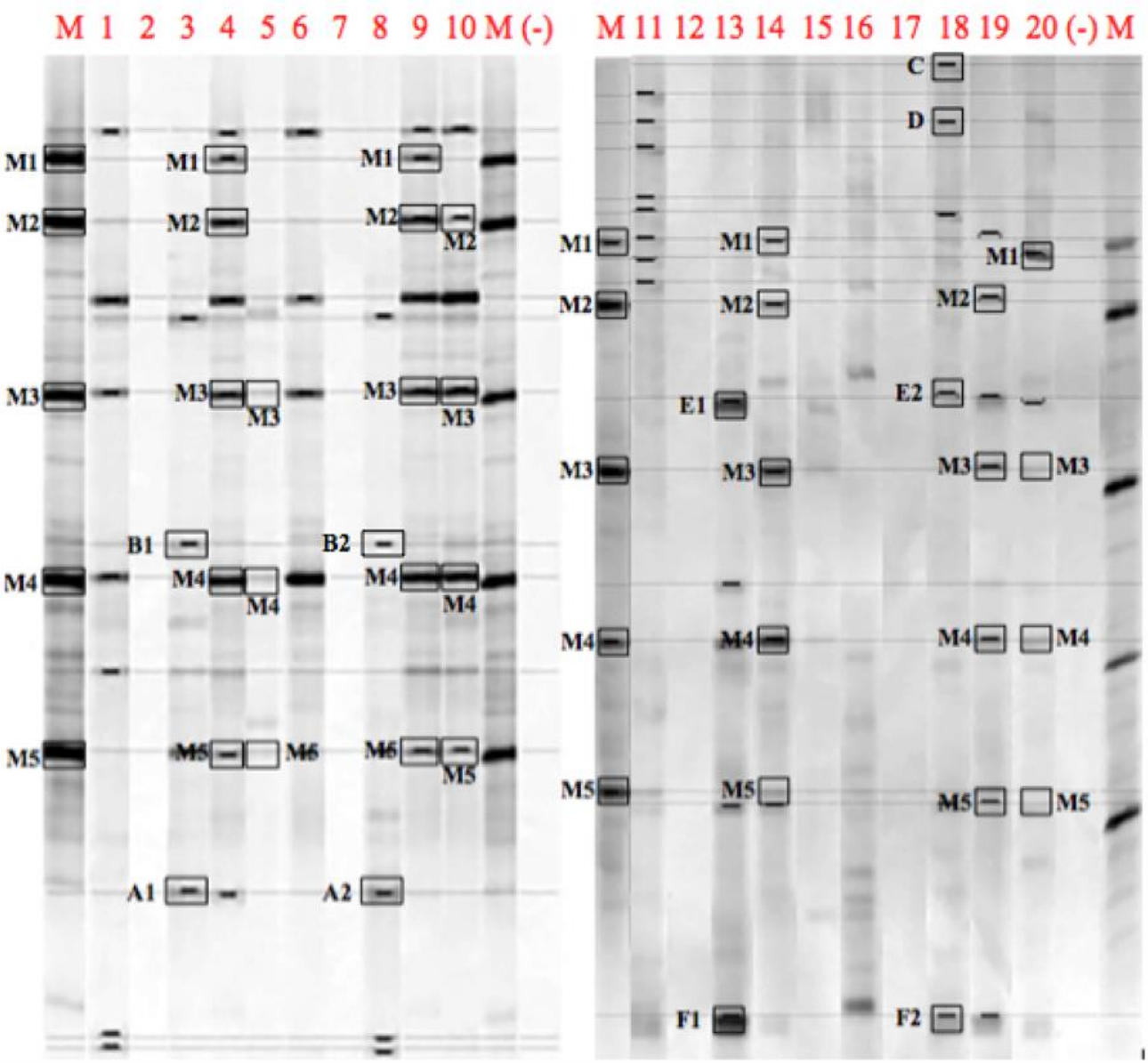
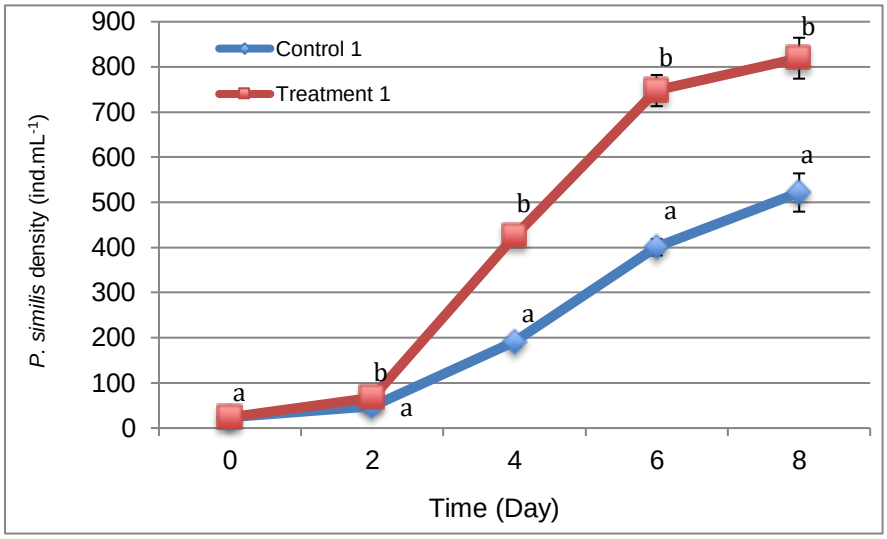


Figure 3.3. DGGE gels (8% acrylamide, 45–60% denaturing gradient) of PCR products with primers 338F-GC and 518R obtained from 1 P Cont & 1 B Cont: non-manipulated MC & unautoclaved algae; 1 P AB & 1 B AB: non-manipulated MC, unautoclaved algae & mixture of four AB; 2 P Cont & 2 B Cont: started in autoclaved water & fed autoclaved algae; 2 P Prob & 2 B Prob: started in autoclaved water, fed autoclaved algae & mixture of 6 species of tentative probiotic bacteria; 2 P Prob+AB & 2 B Prob+AB: started in autoclaved water, fed autoclaved algae & mixture of 6 species of AB killed tentative probiotic bacteria. (Left) DNA from 1 P Cont, treatment 1 P AB, 2 P Cont, treatment 2 P Prob, treatment 2 P Prob+AB on day 6 (columns 1–5) and day 10 (columns 6–10), respectively; (Right) DNA from 1 B Cont, treatment 1 B AB, 2 B Cont, treatment 2 B Prob, treatment 2 B Prob+AB on day 6 (columns 11–15), day 10 (columns 16–20), respectively; M: a marker produced from a template consisting of DNA from pure cultures of different probiotic strains used in the experiment (M1: *L. plantarum strain R22*, M2: *B. subtilis strain* C_{10}, M3: *L. brevis strain* NC_2, M4: *P. acidilactici strain* TC_5 and/or *P. pentosaceus strain* MC_1t_2, M5: *L. fermentum strain* TC_{19}); (-): negative controls.

3.3.6. Effect of probiotic addition on the rotifer performance in large-scale culture

Overall, the population densities were significantly different among treatments, except at the starting point (day 0) (Figure 3.4, rmANOVA, $p<0.001$). The population densities of *P. similis* with the addition of the live mixture of bacteria (treatment 1: 819 ind.mL^{-1}) was significantly higher (57%) than those started in autoclaved water and fed autoclaved algae (control 1: 522 ind.mL^{-1}) at day 8 (t-test, $p<0.05$). The densities of *B. rotundiformis* with the addition of the live probiotic mixture (treatment 1: 205 ind.mL^{-1}) was not significantly different from those started in autoclaved water and fed autoclaved algae (control 2: 186 ind.mL^{-1}) at day 8 (t-test, $p>0.05$).



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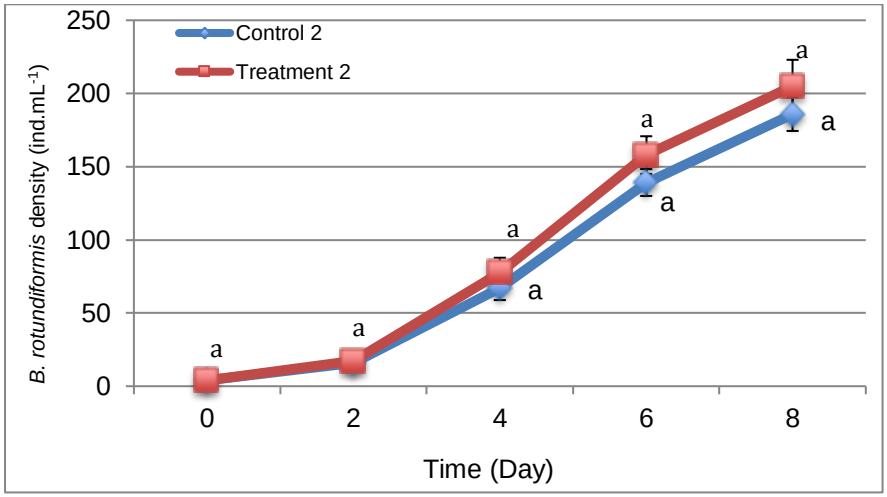

B

Figure 3.4. Growth performance of (A) *P. similis*; (B) *B. rotundiformis* under 2 different culture conditions (mean ± SD, n = 4). Different letters indicate significant differences in each rotifer population density among treatments at each sampling point (t-test).

From day 0 to day 8, growth rates of *P. similis* and *B. rotundiformis* were significantly different among treatments (Table 3.4, one-way ANOVA, $p<0.001$). The growth rate of *P. similis* with the addition of the live probionts (treatment 1: 0.601 day^{-1}) was significantly higher (9 – 14%) than other treatments from both rotifer cultures (Tukey-Kramer, $p<0.05$). By contrast, there were no significant differences in *B. rotundiformis* growth rates where putative probionts were or were not added (treatment 2: 0.552; Control 2: 0.543 day^{-1}) (Tukey-Kramer, $p>0.05$). The maximum growth rate (r-max) of the control 1 was 30.1 – 46.5% lower than those from any other treatment. The results indicated that the *P. similis* was more dependent on the proliferating bacterial community than the *B. rotundiformis* in large-scale culture conditions (Table 3.4).

Table 3.4. *P. similis* and *B. rotundiformis* growth rates (r and r-max) (day^{-1}) in 8 days (from day 0 to day 8) in all treatments (mean ± SD day^{-1}, n=4)

Cultures	Treatments	Growth rate (r)	Maximum growth rate (r-max)
P. similis	Control 1	0.527 ± 0.040^{a}	1.277 ± 0.039^{a}
	Treatment 1	0.601 ± 0.025^{b}	1.578 ± 0.026^{b}
B. rotundiformis	Control 2	0.543 ± 0.046^{a}	1.742 ± 0.179^{b}
	Treatment 2	0.552 ± 0.050^{a}	1.739 ± 0.075^{b}

Control 1, 2: started in autoclaved water, fed autoclaved algae. Treatment 1, 2: started in autoclaved water, fed autoclaved algae & mixture of 6 tentative probiotics. Different letters indicate significant differences in each rotifer population density among treatments at each sampling point (t-test).

3.4. Discussion

The body size, length and width, of *P. similis* were 83 ± 11 µm and 40 ± 6 µm, respectively, 38.1% smaller and 60.3% narrower than *B. rotundiformis*. The best microalgae for the culture of *P. similis* is *N. oculata* (1.5 – 2 µm) and *C. vulgaris* (2 – 6 µm) (Wullur *et al.*, 2009). However, based on the results of this study, the effect of the bacterial community on growth performance of *P. similis* is important.

Rotifer batch cultures have a complex MC and the MC have an important influence on rotifer performance (Miyakawa and Muroga, 1988; Tinh *et al.*, 2006). Therefore, it is important to control or manipulate the MC associated with rotifer cultures to achieve good rotifer production (Planas and Cunha, 1999; Skjermo and Vadstein, 1999; Dhert *et al.*, 2001). In the aquatic environment, only 30% of the bacteria can be found as free living organisms, while the majority (70%) is aggregated around organic substrate,

such as dead organisms, faecal pellets, molts of zooplankton, living phyto- and other zooplankton or around an inorganic core such as clay particles (Seki, 1969; Selmi *et al.*, 2001; Grossart, 2010; Tang *et al.*, 2010; Attramadal *et al.*, 2012b). Bacteria and by extension the microbial loop are mainly considered important as a recycling pathway for C and N in many ecological food webs (Azam *et al.*, 1983). In the rotifer's digestive tract, bacteria can either be digested, be transient or commensal alive microflora. It is clear that bacteria enhance *B. plicatilis* culture performance, either added as pure free living strains (Rombaut *et al.*, 1999; Douillet, 2000) or mixtures of lab cultured bacteria or commercial products (Douillet, 2000), compared to bacteria-free rotifer cultures. Furthermore, *B. plicatilis* sensu stricto had an increased growth performance in the presence of live microbial communities when the rotifers were fed baker's yeast, while there was no effect observed when they were fed *Chlorella*. In this experiment, the addition of the mixture of killed probionts did not have any effect on the growth performance of *B. rotundiformis* culture. Similar results were obtained by Tinh *et al.* (2006), when heat-killed microbial communities were added. As 63% of marine bacteria dry weight is protein, bacteria can be a substantial source of proteins (Simon and Azam, 1989). Depending on the mixture of available food particles, 10 to 40% of the rotifer's diet can consist of bacteria (Arndt, 1993). It was concluded that the rotifer densities and/or egg-to-female ratio dynamics in a eutrophic reservoir were significantly positively correlated with bacterial load (Ooms-Wilms, 1997; López *et al.*, 2007).

In *B. plicatilis* culture, the use of a single (ampicillin at 100 $\mu g.mL^{-1}$, kanamycin at 60 $\mu g.mL^{-1}$, nalidicic acid at 30 $\mu g.mL^{-1}$ and streptomycine at 60 $\mu g.mL^{-1}$) or a mixture of 4 ABs (ampicillin at 100 $\mu g.mL^{-1}$, kanamycin at 60 $\mu g.mL^{-1}$, nalidicic acid at 30 $\mu g.mL^{-1}$ and streptomycine at 60 $\mu g.mL^{-1}$), except chloramphenicol at 40 $\mu g.mL^{-1}$, were earlier found not to affect rotifer reproduction or hatching success and did not show any teratogenic effects (Suga *et al.*, 2011). In this study, the MCs in the *P. similis* and *B. rotundiformis* cultures were treated by the addition of a mixture of four ABs, namely: rifampicin, ampicillin, trimethoprim and gentamycin. No significant differences in hatching percentage were found between the AB free and the AB containing treatments, suggesting that the AB is non-toxic for rotifers. However, in the group 1, the population densities and growth rates of the cultures with addition of AB, with limiting bacterial proliferation (Table 3), were lower than those grown in non-

manipulated MC cultures. It showed that the proliferating bacterial community was a very important factor in the growth performance of both rotifer species, pointing in the direction of the importance of the microbial loop.

At this stage, it is important to mention that in the experiments of group 2, equal number of probionts and *N. oculata* were added to the culture. With an approximated cellular weight for bacteria of 0.2 pg (Heldal *et al.*, 1985) and 10 pg for *N. oculata* (Brown *et al.*, 1993), this means that supplemented probionts constituted around 10% of the food supplied to the cultures. Hence, in cultures in which AB were added (limiting bacterial proliferation, see Table 3), bacteria biomass was only a minor fraction of the total microbial biomass available to the rotifers. In the absence of AB, probionts can proliferate on waste products and constitute a non-quantified amount of the diet of the rotifers. It appears that in the presence of AB, the supplemented probionts do not have a positive nor negative effect on *B. rotundiformis*. Hence, they do not seem to be important for *B. rotundiformis* growth when *N. oculata* is the major food compound. For *P. similis*, the added probionts in the AB treatment seem to have an apparent negative effect. Keeping in mind that they only constitute a minor fraction of the total diet, it can be suggested that the added probionts do not constitute as such an important source of nutrients. In addition due to the AB presence, regrowth of bacteria and probionts (see Table 3) is limited, probably limiting *P. similis* growth (this interpretation assumes that the presence of active metabolizing bacteria, namely the standing MC in combination with the probionts, are beneficial for *P. similis* growth). As such, the effect of AB in the *P. similis* experiment of group 1 and group 2 seem to be similar, as limiting bacterial growth strongly suppresses growth. In the absence of AB, the addition of probionts has a major effect on both *B. rotundiformis* and *P. similis*. Here, it is important to compare growth relative to the control with and without AB. In comparison to the control without AB, the results seem to indicate that the proliferation of probionts in the cultures (relative a non-manipulated control) is very beneficial which can be due to the combination of additional bacterial biomass available which can have an additional specific probiotic effect. The data, however, do not allow to quantitatively distinguishing between a pure nutritional effect and a probiotic effect. The data do indicate that especially for *P. similis* the added probionts under conducive conditions for regrowth, are very beneficial for *P. similis* and do not have an anti-nutritional effect (as could be concluded from the reduced growth rate of *P. similis*

when probionts and AB are added together). Also, under the same culture conditions, the bacterial densities on TCBS, MRS and MA were always lower in *P. similis* cultures than those from *B. rotundiformis* at day 10. Acknowledging that not all colonies growing on TCBS are actually *Vibrio*, these results seem to indicate that *P.similis* culture contain less *Vibrio*, an interpretation that would need confirmation by for instance 16S rDNA sequencing of individual colonies. The overall reduced bacterial counts indicate that *P. similis* can apparently ingest and digest the bacteria. In comparison with the control with AB it becomes clear that the growth performance of the rotifer *P. similis* is more dependent on the proliferating bacterial community than the rotifer *B. rotundiformis.* This is the new finding of this study, as there was no report so far about the relative importance of bacterial community for different rotifer species.

The results from the DGGE analysis of MC in rotifer culture (rotifers and water) in the controls and treatments showed that bands from the *L. plantarum* (band M1), *B. subtilis* (band M2), *L. brevis* (band M3), *P. acidilactici* (band M4), *P. pentosaceus* (band M4) and *L. fermentum* (band M5) were dominant in the treatments started in autoclaved water, fed autoclaved algae with the addition of the mixture of six species of tentative live or killed probiotic bacteria at day 6 and day 10. On the other hand, these from LAB and *B. subtilis* were not present in other controls and treatments without the addition of probiotic bacteria. It explains that the LAB and *B. subtilis* could be absorbed by rotifers. Hence, this could give positive effects when feeding these rotifers to the fish larvae (to be tested).

In conclusion, addition of proliferating bacteria, for example, live LAB and *B. subtilis*, from the beginning of *P. similis* and *B. rotundiformis* cultures, at the density of 10^6 $CFU.mL^{-1}.species^{-1}.day^{-1}$, enhances the rotifer production, alters the MC composition and allows shortening the culture period to 6 – 8 days, hence reduce the chance of protozoan contamination (which is one of the main reasons for the sudden collapse of *P. similis* culture), compared to 11 days as reported by Wullur et al. (2009). The *P. similis* takes more advantage from proliferating MC than the *B. rotundiformis.*

CHAPTER 4

Larviculture of rabbit fish *(Siganus guttatus)*

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Abstract

The rabbit fish (*S. guttatus*) is an increasingly popular species to cultivate in Vietnam. However, larviculture of rabbitfish is still unsuccessful, due to the lack of proper first feeding food for the larvae. In this study, different foods for rabbit fish larval rearing from hatching to day 25 were tested. The data generated from this study will provide valuable information in order to make an accurate assessment of the effects of nutrition on survival and growth rate of rabbit fish larvae. In the first experiment, *P. similis* and *B. rotundiformis* were tested as initial food for larvae. The data showed that the smaller rotifer *P. similis* was by far the better starter food for rabbit fish larvae than the bigger rotifer *B. rotundiformis.* The feeding incidence was earlier and significantly higher survival rates were obtained in the treatments fed *P. similis,* than those fed *B. rotundiformis.* The second experiment showed the importance of the SS-type *B. rotundiformis* on the survival and the total length of rabbit fish larvae when introduced into the culture system from day 6, which provided the best survivals, compared to the survival of larvae fed *B. rotundiformis* at other time points. Furthermore, the feeding of probiotic-enriched rotifers to the larvae did not have any effect on the ingestion of rotifers, such as first feeding incidence, feeding percentage at 36 and 96 hph and the amount of *P. similis* and *B. rotundiformis* in the gut. However, the results of this test showed that the larvae fed probiotic-enriched rotifers had significantly higher survival than the larvae fed non-enriched rotifers. Finally, the effect of different rearing protocols on the performance and survival of larvae from day 10 to 25 has also been tested. The results showed that rotifers still play an important role as food to the fish larvae beyond day 10. After that, *Artemia* was most suitable food to the larvae. The feeding schemes including *Artemia* and rotifers resulted in significantly higher survival and total length of larvae than the feeding scheme without *Artemia.* Compound diet was good for larvae when combined with rotifers and *Artemia* (Vinh Chau, Vietnam).

Keywords: Rabbit fish, *Siganus guttatus*, larviculture; larval rearing; probiotics.

4.1. Introduction

Rabbit fish *(S. guttatus)* belongs to the genus *Siganus* in the family Siganidae (Lam, 1974). The cultivation of rabbit fish is increasingly popular in Southeast Asia and especially in Central Vietnam as there is an increasing demand from consumers (Hara *et al.*, 1986a) and this species has a low trophic level, as they can be fed seaweed. However, lack of year-round-fry supply is one of the critical problems for farmers wanting to expand their rabbit fish culture (Hara *et al.*, 1986b).

The male rabbit fish becomes sexually mature at 10 months of age, while the female matures later, at around 12 months of age (Juario *et al.*, 1985). The rabbit fish can spawn all year round (Hara *et al.*, 1986a; Hara *et al.*, 1986b). *S. guttatus* have no difficulties to spawn in captivity. Fish that are subjected to stress (such as handling) can still spawn without any need for hormonal treatment (Ayson, 1989). The eggs hatch after approximately 20 h. When rabbit fish larvae hatch, they are transparent with an approximate length of 1.7 mm (Hara *et al.*, 1986a; Rachmansyah *et al.*, 2007). During this early period, the larvae show high sensitivity to external stress and handling, which results in low survival. It is very important that the larvae of rabbit fish have an extra small size of food to be ingested (Hara *et al.*, 1986b; Ayson, 1989). This has a great impact on the growth and survival of the fry. Up to day 6 after the larvae have hatched, most rotifers are still too big for the mouth of the fry. This causes big problems for farmers rearing fry of rabbit fish. Kohno *et al.* (1988) showed that ingestion of exogenous food started when the *S. guttatus* mouth had a size of 200 µm, which was 55.5 h after hatching. The ingested rotifers had a width of 125 µm. The width of the ingested rotifers was 55 – 70% of the width of the *S. guttatus* mouth. One commonly used rotifer is *B. plicatilis* which is fed to the larvae when the fry start eating exogenous food (Watanabe and Kiron, 1994). This species is approximately 90 to 340 µm and is therefore expected to be a suitable food to *S. guttatus*. Kohno *et al.* (1988) showed that high mortality of *S. guttatus* fry occurred at different growth stages and when changing from endogenous to exogenous food.

In this study, different foods for rabbit fish larval rearing from hatching to day 25 were tested. The data generated from this study provide valuable information on the effect of feeding strategies on rabbit fish survival and growth, and could be found essential in the establishment of industrial rabbit fish larviculture protocols.

4.2. Materials and methods

4.2.1. Brood stocks and larval preparation

Selected brood stocks were paired in a 2 m^3 circular spawning tank. The females were induced with 2 IU of HCG.g^{-1} body weight every 24h until spawning. The males were not treated with hormones. Fertilized eggs attached to the plastic substrate were transferred to a 500L-hatching tank. After 20 hph, the number of newly hatched larvae was estimated and distributed to rearing tanks for experiments.

4.2.2. Food preparation

The rotifers *P. similis* (Okinawa strain, Japan), *B. rotundiformis* (Bali strain, Indonesia), *B. plicatilis* (Mie strain, Japan) were obtained from the Lab. of Aquaculture Biology, Nagasaki University, Japan. The rotifers were cultured at 28 ± 1°C in 400L fiberglass tank, and were fed *N. oculata* (strain CCMP525, Reed Mariculture, USA). The brine shrimp *A. franciscana* (Vinh Chau strain, Vinh Chau company, Vietnam) was hatched in 32 g.L^{-1} seawater and instar I nauplii were rinsed in seawater prior to feeding to the larvae. The compound diet used in the experiment was from C.P. Group (C.P. – 9000, 0.4 – 0.6 mm in diameter, 42% crude protein). The whole sample of food was used for FAME analysis at the Lab. of Aquaculture & Artemia Reference Center, Ghent University. The FAME analysis followed the modified procedure of Lepage and Roy (1984), and conducted as described previously by Coutteau *et al.* (1995) (Table 4.1).

4.2.3. Experimental design

Experiment 1 (First feeding experiment): The aim of the experiment was to determine whether rabbit fish larvae could survive till 240 hph (10 days) under given feeding schemes. This experiment was conducted in 400L fiberglass tanks, containing 300L of seawater (32 g.L^{-1}) at 28 ± 1°C, pH = 8 ± 0.5. The larval density was 20 larvae.L^{-1}. The photoperiod was maintained at 12L:12D throughout the experiment. The seawater was filtered, ozonized and de-ozonized before pumping to the tank. During the experiment, the rearing water was not exchanged and an air stone placed at the bottom center of each tank provided aeration; 1 control and 2 feeding schemes are described in Table 4.2. Rotifers were fed to the larvae twice a day at 8 AM and 4 PM. The rotifers in each tank were counted before adding new rotifers (top up) to maintain the same dry weight and encounter rate. Each treatment was conducted in triplicate. Ten larvae from each replicate were collected at 36, 48, 60, 96, 168 and 216

hph for the analysis of feeding incidence, percentage of feeding larvae and amount of rotifers in the gut using the method from Wullur *et al.* (2009; 2011). Feeding incidence and rotifer quantity in the gut was determined by dissecting the gut of the larvae under a stereomicroscope. The number of rotifers was counted based on the number of mastax (calcified jaw), which cannot be digested by the larvae (Akazawa *et al.*, 2008). Ten days after hatching, all remaining larvae from each replicate were collected at the end of the experiment (240 hph) for survival calculation.

Table 4.1. Fatty acids (% of composition) of food used in experiments

Fatty acids	*P. similis*	*B. rotundiformis*	*B. plicatilis*	*Artemia*	Compound diet
14:0	4.03	1.10	1.52	2.16	3.64
14:1(n-5)	0.97	1.13	0.98	0.95	0.12
16:0	17.42	14.05	12.81	12.35	24.41
16:1(n-7)	15.07	5.86	7.72	12.20	3.26
18:0	7.38	4.22	3.62	3.52	4.13
18:1(n-9)	7.92	2.34	3.31	14.85	13.09
18:1(n-7)	7.27	3.98	3.62	9.80	2.30
18:2(n-6)	2.44	5.55	4.49	3.58	12.44
18:3(n-6)	0.14	0.09	0.05	0.46	0.05
18:3(n-3)	0.53	2.56	2.87	2.22	3.31
18:4(n-3)	0.41	0.48	0.71	1.02	1.27
20:1(n-9)	0.40	1.27	1.12	0.27	4.17
20:1(n-7)	2.23	0.87	0.72	0.17	0.24
20:3(n-6)	0.27	1.69	1.82	0.19	0.10
20:4(n-6)	2.33	4.41	5.35	2.74	0.63
20:4(n-3)	2.25	1.56	1.34	0.36	0.32
22:0	1.27	0.52	0.49	0.21	0.22
20:5(n-3)	6.00	4.51	6.44	11.58	4.94
22:5(n-3)	2.62	3.89	3.42	0.04	0.47
22:6(n-3)	0.00	0.00	0.00	0.09	10.40
Others	24.05	42.15	40.10	24.44	24.65
Total	100	100	100	100	100
DHA/EPA	0.00	0.00	0.00	0.01	2.11

Table 4.2. Description of the feeding schemes from hatching to 240 hph

Treatments	Description of feeding schemes
Control	Starvation control
Treatment 1	*P. similis* only (1 mg DW.L^{-1}.day^{-1}~ 29 ind.mL^{-1}.day^{-1})
Treatment 2	*B. rotundiformis* only (1 mg DW.L^{-1}.day^{-1} ~ 5.6 ind.mL^{-1}.day^{-1})

Experiment 2 (Optimization of rearing protocol from hatching to 240 hph): Based on the results obtained in experiment 1, a second experiment to optimize the rearing protocol from hatching to 240 hph (10 days) was conducted. Basically, the timing of the introduction of *B. rotundiformis,* with a background feeding of *P. similis,* was tested. The same tanks, water volume, temperature, salinity, pH, photoperiod, water exchange and aeration were applied as described in experiment 1; 2 controls and 4 feeding treatments are described in Table 4.3. The aims of this experiment were to determine a suitable feeding scheme resulting in the highest larval survival at 240 hph and the highest growth rate during the first 240 hph. In this experiment, the non-fed treatment served as a negative control (control 1). The best feeding scheme from experiment 1 (*P. similis*) was used as positive control (control 2). In the other four treatments, the addition of *B. rotundiformis* started on day 5 (treatment 1), 6 (treatment 2), 7 (treatment 3) or 8 (treatment 4). One mg.L^{-1} DW of rotifer was added daily for all treatments until the end of the experiment. Before *B. rotundiformis* was added, the ration of *P. similis* was maintained at 1 mg DW per day. When *B. rotundiformis* was added, the amount of *P. similis* was reduced to 0.5 mg DW per day, combining with 0.5 mg DW per day of *B. rotundiformis.* Rotifers were provided to the fish larvae twice a day at 8AM and 4PM. The rotifers in each tank were counted before adding new rotifers (top up) to maintain the same dry weight and encounter rate. Each treatment was conducted in triplicate. Ten larvae from each replicate were collected at 36, 96, 120, 168, 192 and 216 hph for analyzing the feeding incidence, percentage of feeding larvae and amount of rotifers in the gut (Wullur *et al.*, 2011) as described in the section 4.2.3.1. A hundred larvae from each replicate were collected at 240 hph for larval total length measurement using the Optika Vision 2.0 (Ponteranica, Italy). All remaining larvae from each replicate were collected at 240 hph for defining the survival.

Table 4.3. Description of feeding schemes from hatching to 240 hph

Treatments	Description of the feeding scheme
Control 1	Starvation control
Control 2	*P. similis* (1 mg DW.L^{-1}.day^{-1} ~ 29 ind.mL^{-1}.day^{-1})
Treatment 1	Control 2 (DW of *P. similis* reduces by half from day 5) + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} ~ 2.8 ind.mL^{-1}.day^{-1} from day 5)
Treatment 2	Control 2 (DW of *P. similis* reduces by half from day 6) + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} ~ 2.8 ind.mL^{-1}.day^{-1} from day 6)
Treatment 3	Control 2 (DW of *P. similis* reduces by half from day 7) + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} ~ 2.8 ind.mL^{-1}.day^{-1} from day 7)
Treatment 4	Control 2 (DW of *P. similis* reduces by half from day 8) + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} ~ 2.8 ind.mL^{-1}.day^{-1} from day 8)

Experiment 3 (Effect of the addition of the mixture of LAB and *B. subtilis* on the performance and survival of larvae from hatching to 240 hph): The aim of this experiment was to determine whether probiotic-enriched rotifers play an important role in the performance of rabbit fish larvae till 240 hph (10 days). The experimental design was identical to experiment 1 described above. Two feeding schemes were conducted as mentioned in Table 4.4. Rotifers were provided to the larvae twice a day (8AM & 4PM). The rotifers in each tank were counted before adding new rotifers (top up) to maintain the same dry weight and encounter rate. Each treatment was conducted in 4 replicates. In treatment 2, both rotifers were enriched with a mixture of *L. plantarum, L. fermentum, L. brevis, P. acidilactici, P. pentosaceus* and *B. subtilis* (10^6 CFU.mL^{-1}.strain^{-1}, totally 6 x 10^6.CFU.mL^{-1}) following the method described in chapter 2. From each replicate, 10 larvae were collected at 36, 96, 120, 144, 168, 216 hph for the analysis of feeding incidence, % of feeding larvae and amount of rotifers in gut using the method from Wullur *et al.* (2011) as described in the section 4.2.3.1. At 240 hph, 100 larvae of each replicate were collected for length measurement (Optika Vision 2.0, Ponteranica, Italy) and all remaining larvae from each replicate were collected for survival calculation.

Table 4.4. Feeding schemes used in two treatments from hatching to 240 hph

Treatments	Description of the feeding scheme
Treatment 1	Non-probiotic-enriched *P. similis* (1mg DW.L^{-1}.day^{-1} ~ 29 ind.mL^{-1}.day^{-1} before *B. rotundiformis* added, 0.5 mg DW.L^{-1}.day^{-1} ~ 14.5 ind.ml^{-1}.day^{-1} after *B. rotundiformis* added) + non-probiotic-enriched *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} ~ 2.8 ind.mL^{-1}.day^{-1} starting on day 6)
Treatment 2	Same as treatment 1, but probiotic-enriched rotifers were fed to the larvae

Experiment 4 (Effect of different feeding schemes on the performance and survival of larvae from day 10 to day 25): The aim of this experiment was to determine which feeding regime resulted in the highest larval survival from day 10 to 25. The experiment was conducted in the same tank size, water volume, temperature, salinity, pH, photoperiod and aeration as described in experiment 1. Four feeding trials were described (Table 4.5). The larval density was 118 larvae.tank^{-1}. Rotifers and *Artemia* instar I were fed to the larvae twice a day at 8AM and 4PM. The rotifers and *Artemia* in each tank were counted before adding new rotifers and *Artemia* instar I (top up) to maintain the same dry weight and encounter rate. Compound diet was fed to the larvae 4 times.day^{-1} at 6AM, 10AM, 2PM and 6PM. Each treatment was conducted in triplicate. Three larvae from each replicate were collected daily from day 10 to 13 for the analysis of feeding incidence, percentage of feeding larvae under a

stereomicroscope. All remaining larvae from each replicate were collected at the end (day 25) for survival calculation and larval length measurement (Optika Vision 2.0, Ponteranica, Italy).

Table 4.5. Feeding schemes used in four treatments from day 10 to day 25

Treatments	Description of the feeding scheme
Treatment 1	*Artemia* instar I + *B. plicatilis* (1 g DW.tank^{-1}.day^{-1} each)
Treatment 2	Compound diet + *B. plicatilis* (1 g DW.tank^{-1}.day^{-1} each)
Treatment 3	*Artemia* instar I + compound diet (1 g DW.tank^{-1}.day^{-1} each)
Treatment 4	*Artemia* instar I + *B. plicatilis* + compound diet (2/3 g DW.tank^{-1}.day^{-1} each)

4.2.4. Data analysis

All data that present % values have been arcsine transformed for statistical analysis. The Skewness/Kurtosis test was used to check the normal distribution of dataset. If the dataset was normal distributed, the significant differences between means in feeding incidence, percentage of feeding larvae, amount of rotifers in gut, larval length and survival at each sampling point between treatments were detected by one-way ANOVA, followed by the Tukey-Kramer post-tests for multiple comparisons. Student t-test was used where applicable. If the dataset was not normal distributed, the Kruskal-Wallis and Wilcoxon tests were used for multiple comparisons. The statistical significance of the differences of the standard deviations of larval length among treatments was detected by F-Fisher test. The significant levels of 0.05 ($p \leq 0.05$) indicate that the differences between some of the standard deviations are statistically significant. All tests were run by Microsoft Excel 2010, SPSS 22.0 and STATA 13.1.

4.3. Results

4.3.1. Experiment 1: First feeding experiment

Feeding incidence and percentage, amount of rotifers in larval gut content: At each sampling time point, the percentage of larvae feeding on rotifers was significantly different among treatments, except at 96 hph (student t-test, $p<0.05$) (Table 4.6). The first feeding incidence of larvae was at 36 hph in the treatment with larvae fed *P. similis* (treatment 1: 57%) and at 60 hph in the treatment with larvae fed *B. rotundiformis*, which was 53% at treatment 2. The percentage of feeding larvae was 100% at 48 hph (larvae fed *P. similis*) and at 96 hph (larvae fed *B. rotundiformis*). From 36 to 216 hph, the amount of rotifers in larval gut in the larvae fed *P. similis* increased from 44 to 578 ng DW of rotifers per larvae. From 60 to 216 hph, the rotifer

ingestion by the larvae fed *B. rotundiformis* increased from 169 to 2252 ng DW of rotifers per larvae. From 96 to 216 hph, the *B. rotundiformis* ingestion by the larvae was higher than those fed *P. similis* (84% at 96 hph, 390% at 216 hph) (Table 4.7).

Survival at 240 hph: The survival was significantly different among treatments (one-way ANOVA, $p<0.001$). In the starvation control, all larvae died between 72 – 96 hph. The survival of larvae fed *P. similis* was 7.2 ± 0.4%, which was significantly higher (167%) than those fed *B. rotundiformis* (2.7 ± 0.1%) (Tukey Kramer, $p<0.05$).

Table 4.6: Feeding incidence and % (mean ± S.D.) of fish larvae that ate at sampling points (36, 48, 60 and 96 hph) in all treatments (student t-test, different letters from each column indicate significant differences among treatments on the same sampling point, n=3).

Treatments*	Feeding incidence	Feeding % 36 hph	Feeding % 48 hph	Feeding % 60 hph	Feeding % 96 hph
Treatment 1	36 hph	57 ± 15^{b}	100 ± 0^{a}	100 ± 0^{a}	100 ± 0^{a}
Treatment 2	60 hph	0 ± 0^{a}	0 ± 0^{a}	53 ± 15^{b}	100 ± 0^{a}

Table 4.7. Amount of rotifers (mean ± S.D.) in the larval gut at sampling points (36, 48, 60, 96, 168 and 216 hph) in all treatments (student t-test, different letters from each column indicate significant differences among treatments on the same sampling point, n=3).

Treatments*	The weight of rotifers in the larval gut content (ng DW.gut^{-1})					
	36 hph	48 hph	60 hph	96 hph	168 hph	216 hph
Treatment 1	44 ± 11^{b}	123 ± 11^{b}	171 ± 9^{a}	235 ± 13^{a}	431 ± 17^{a}	578 ± 77^{a}
Treatment 2	0 ± 0^{a}	0 ± 0^{a}	169 ± 68^{a}	433 ± 86^{b}	1788 ± 169^{b}	2252 ± 347^{b}

Treatment 1: larvae fed *P. similis* (1 mg DW.L^{-1}.day^{-1}). Treatment 2: larvae fed *B. rotundiformis* (1 mg DW.L^{-1}.day^{-1}).

4.3.2. Experiment 2: Optimization of rearing protocol from hatching to 240 hph

Feeding incidence, % of feeding larvae and amount of rotifers in larval gut: The first feeding incidence of larvae on rotifers in all treatments, except the starvation control, was observed at 36 hph. At 36 hph, there was no significant difference in the % of feeding larvae in all treatments (Table 4.8). At 36 hph, the % of feeding larvae ranged from 73 (treatment 1) to 80 (control 2, treatment 2 and 3). At 96 hph, the % of feeding larvae was 100% in all treatments (ANOVA, $p>0.05$), except the starvation control (no feeding). The amount of rotifers (either *P. similis* or *B. rotundiformis*) in the larval gut content was shown in Table 4.9. At 36 and 96 hph, there was no significant difference in the amount of *P. similis* in the larval gut in all treatments (Tukey-Kramer, $p<0.05$). However, after *B. rotundiformis* was introduced (from 144 hph), the amount of *P. similis* in the larval gut reduced by 19 – 41% as the larvae started to feed predominantly on *B. rotundiformis*. There was no significant difference in the amount

of *B. rotundiformis* in the larval gut in all treatment at each sampling points. At 120 hph, the amount of *B. rotundiformis* in the larval gut was 552 ng DW.gut^{-1} in treatment 1. At 216 hph, the amount of *B. rotundiformis* in the larval gut ranged from 2139 ng DW.gut^{-1} in treatment 2 to 2258 ng DW.gut^{-1} in treatment 3 (Tukey-Kramer, $p>0.05$). At 120 hph, the total amount of rotifers ingested by larvae co-fed both rotifers was 115 – 149% higher than those still fed solely *P. similis*, and the difference increased to 270 – 390% at 216 hph in the total amount of rotifers ingested by the larvae co-fed both rotifers (treatment 1 to 4) and those fed solely *P. similis* (control 2).

Table 4.8. Feeding incidence and % (mean ± S.D., n=3) of larvae that ate at sampling points (36 and 96 hph) in all treatments (one-way ANOVA, Tukey-Kramer tests for multiple comparisons, different letters from each column indicate significant differences among treatments at same sampling point)

Treatments*	First feeding incidence	Feeding % 36 hph	Feeding % 96 hph
Control 2	36 hph	80 ± 6^{a}	100^{a}
Treatment 1	36 hph	73 ± 6^{a}	100^{a}
Treatment 2	36 hph	80 ± 6^{a}	100^{a}
Treatment 3	36 hph	80 ± 10^{a}	100^{a}
Treatment 4	36 hph	70 ± 20^{a}	100^{a}

Table 4.9. The amount of rotifers (mean ± S.D.) in the larval gut content at sampling points (36, 96, 120, 144, 168, 192 and 216 hph) in all treatments (one-way ANOVA, Tukey-Kramer tests for multiple comparisons, different letters from each column indicate significant differences in the weight of each rotifers in the larval gut content among treatments at the same sampling point, n=3) and number of stars from each column indicate significant differences in the weight of both rotifers combined in the larval gut content among treatments at the same sampling point, n=3)

Food	Treatment	The weight of rotifers in the larval gut content (ng DW.gut-1)						
		36 hph	96 hph	120 hph	144 hph	168 hph	192 hph	216 hph
P. similis	Control 2	54 ± 10^{a}	274 ± 30^{b*}	354 ± 33^{a*}	379 ± 27^{b*}	431 ± 40^{b*}	526 ± 45^{b*}	634 ± 54^{b*}
	Treatment 1	56 ± 8^{a}	261 ± 19^{b}	329 ± 56^{a}	267 ± 25^{a}	210 ± 37^{a}	217 ± 25^{a}	210 ± 20^{a}
	Treatment 2	51 ± 19^{a}	245 ± 38^{b}	395 ± 24^{b}	269 ± 18^{a}	227 ± 39^{a}	235 ± 37^{a}	194 ± 20^{a}
	Treatment 3	56 ± 13^{a}	264 ± 16^{b}	391 ± 25^{b}	397 ± 30^{b}	221 ± 26^{a}	223 ± 19^{a}	222 ± 16^{a}
	Treatment 4	53 ± 9^{a}	249 ± 22^{b}	410 ± 25^{b}	393 ± 32^{b}	422 ± 38^{b}	247 ± 31^{a}	203 ± 20^{a}
B. rotundiformis	Treatment 1	-	-	552 ± 47^{c}	1123 ± 60^{c}	1644 ± 110^{c}	1807 ± 86^{c}	2177 ± 97^{c}
	Treatment 2	-	-	-	1085 ± 97^{c}	1537 ± 95^{c}	1719 ± 76^{c}	2139 ± 122^{c}
	Treatment 3	-	-	-	-	1556 ± 66^{c}	1662 ± 85^{c}	2258 ± 94^{c}
	Treatment 4	-	-	-	-	-	1700 ± 128^{c}	2145 ± 68^{c}
Both rotifers combined	Control 2	54 ± 10*	274 ± 30*	354 ± 33*	379 ± 27*	431 ± 40*	526 ± 45*	634 ± 54*
	Treatment 1	56 ± 8*	261 ± 19*	881 ± 86**	1390 ± 81**	1854 ± 127**	2024 ± 111**	2387 ± 82**
	Treatment 2	51 ± 19*	245 ± 38*	395 ± 24*	1354 ± 115**	1766 ± 100**	1954 ± 77**	2333 ± 114**
	Treatment 3	56 ± 13*	264 ± 16*	391 ± 25*	397 ± 30*	1777 ± 87**	1886 ± 98**	2480 ± 88**
	Treatment 4	53 ± 9*	249 ± 22*	410 ± 25*	393 ± 32*	422 ± 38*	1947 ± 149**	2348 ± 73**

Treatment 1: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 5). Treatment 2: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 6). Treatment 3: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 7). Treatment 4: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 8).

Survival at 240 hph: The survival was significantly different among treatments (Figure 4.1, one-way ANOVA, $p<0.001$). The survival in the treatment where *B. rotundiformis* was added on day 6 was significantly higher than in any other treatment (13.7%) (Tukey-Kramer, $p<0.05$), which was 94% higher than those without *B. rotundiformis* addition and 69% higher than those with *B. rotundiformis* added on day 8.

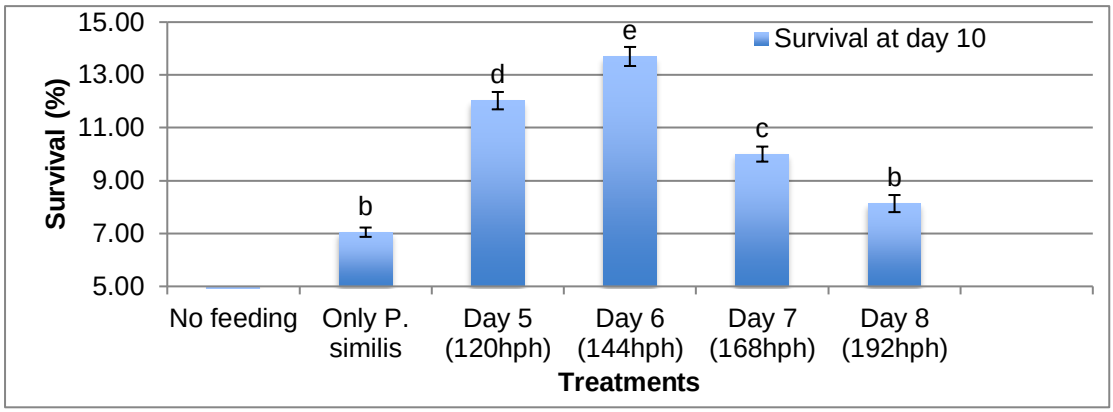

Figure 4.1. Survival (%) at 240 hph (mean ± S.D.) in all treatments (one-way ANOVA, Tukey-Kramer tests for multiple comparisons, different letters indicate significant differences in survival at 240 hph, n=3). No feeding: starvation control. Only *P. similis*: fed *P. similis* (1 mg DW.L^{-1}.day^{-1}). Day 5: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 5). Day 6: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 6). Day 7: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 7). Day 8: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 8).

The larval length at 240 hph: The larval length (mean ± S.D.) was significantly different among treatments at 240 hph (Kruskal-Wallis, $p=0.011$) (Table 4.10). There were no significant differences in the larval length of treatment 1 (*B. rotundiformis* added from day 5, 3.69 mm), treatment 2 (*B. rotundiformis* added from day 6, 3.67 mm), treatment 3 (*B. rotundiformis* added from day 7, 3.62 mm) and treatment 4 (*B. rotundiformis* added from day 8, 3.59 mm) and control 2 (no *B. rotundiformis* added, 3.37 mm) (Kruskal-Wallis, $p>0.05$), except those between treatment 1 and control 2 (Kruskal-Wallis, $p<0.05$) (Table 4.10). The larval length in control 2 and all treatments had no normal distribution. The larval length in treatment 2 was a right-skewed distribution (skewness coefficient (SC) = 0.04), while the larval length in control 2 (SC = -0.08), treatment 1 (SC = -0.02), treatment 3 (SC = -0.16) and (treatment 4, SC = -0.39) were left-skewed distributions (Skewness/Kurtosis). There were no significant differences in the standard deviations of larval length in the control 2 and all treatments (F-Fisher, $p>0.05$), except between treatment 1 (0.140 ± 0.002 mm) and control 2 (0.160 ± 0.008 mm) ($p=0.0055$), treatment 1 and 2 (0.160 ± 0.007 mm) ($p=0.0044$). The larval length distributions in treatment 1 were significantly sharper than those obtained in control 2 and treatment 2 (Figure 4.2).

Table 4.10. Average larval length (mm, mean ± S.D., n=3) at 240 hph in all treatments. Different letters from the same row indicate significant differences in the larval length at 240 hph between treatments)

Treatments	Control 2	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Larval length (mm)	3.37 ± 0.01[a]	3.69 ± 0.02[b]	3.67 ± 0.01[b]	3.62 ± 0.02[b]	3.59 ± 0.02[b]

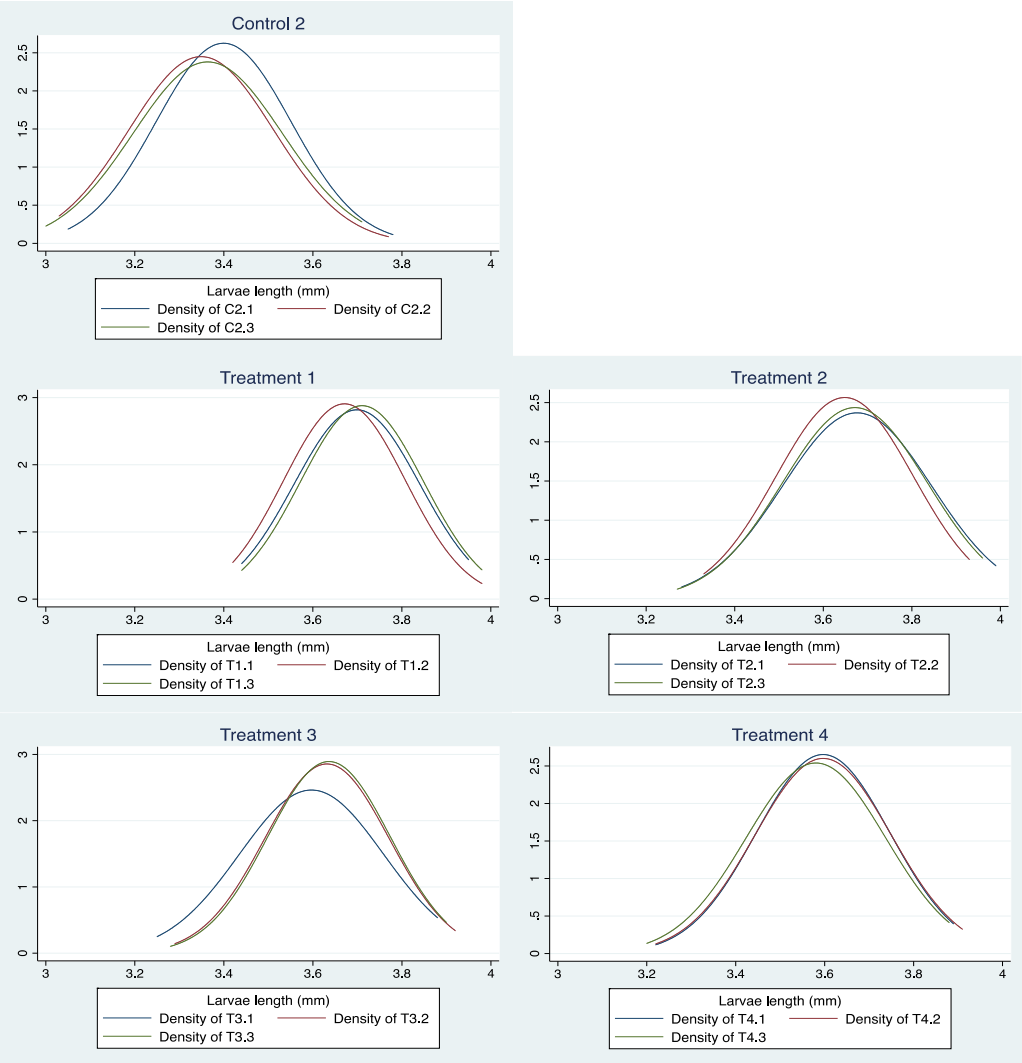

Figure 4.2. Larval length distributions from 3 replicates in control and treatments at 240 hph.

Control 2: fed *P. similis* (1mg DW.L^{-1}.day^{-1}). Treatment 1: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 5). Treatment 2: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 6). Treatment 3: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 7). Treatment 4: control 2 + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1} from day 8).

4.3.3. Experiment 3: Effect of the addition of LAB and *B. subtilis* on the performance and survival of larvae from hatching to 240 hph.

Feeding incidence and % of feeding larvae of fish according to rearing time: The first feeding incidence was observed at 36 hph in both treatments and the % of feeding larvae ranged from 70% in treatment 1 to 73% in treatment 2. From 96 hph, all larvae were feeding in both treatments (t-test, p>0.05) (Table 4.11).

Amount of rotifers in the gut: There were no significant differences in the amount of *P. similis* in larval gut between treatments at each sampling point (t-test, p>0.05). At 168 hph, the amount of *P. similis* in the gut of both treatments decreased. There were no significant differences in the rotifer amount in gut between treatments at each sampling point (treatment 1: 49 – 2268 ng DW.gut^{-1} at 36 & 216 hph, respectively; treatment 2: 52 – 2363 ng DW.gut^{-1} at 36 and 216 hph, respectively) (t-test, p>0.05) (Table 4.12).

Survival at 240 hph: The survival of larvae in treatment fed probiont-enriched rotifers (16.1 ± 0.7%) was 22% higher than those fed non probiont-enriched rotifers (13.2 ± 0.4%) (t-test, p<0.05).

The larval length: At 240 hph, the larval length (mean ± S.D.) in treatment 2 (3.64 ± 0.03 mm) was significantly higher than those from treatment 1 (3.53 ± 0.01 mm) (Wilcoxon, p>0.05). The larval length in both treatments had no normal distribution. The larval length in treatment 2 (SC = -0.58) and 1 (SC = -0.09) had left-skewed distributions (Skewness/Kurtosis). Curve analysis of larval length revealed a significantly higher standard deviation in treatment 1 (0.22 ± 0.014 mm), compared to those obtained in treatment 2 (0.19 ± 0.028 mm) at 240 hph (F-Fisher, p=0.0023). It showed that the curve of larval length observed in treatment 2 was significantly sharper than those obtained in treatment 1 (Figure 4.3).

Table 4.11. Feeding incidence and percentage of feeding larvae (mean ± S.D.) of fish larvae in all treatments at 36 and 96 hph (student t-test, different letters from each column indicate significant differences in percentage of feeding larvae among treatments at the same sampling point, n=4),

Treatments	Feeding incidence	Feeding % at 36 hph	Feeding % at 96 hph
Treatment 1	36 hph	70 ± 8^{a}	100 ± 0^{a}
Treatment 2	36 hph	73 ± 10^{a}	100 ± 0^{a}

Table 4.12. Amount of rotifers (mean ± S.D.) in larval gut content at sampling points (36, 96, 144, 168 and 216 hph) in all treatments (student t-test, different letters from each column indicate significant differences among treatments at the same sampling point, n=4)

Food	Treatments*	The amount of *P. similis* in the larval gut content (ind.gut^{-1})				
		36 hph	96 hph	144 hph	168 hph	216 hph
P. similis	Treatment 1	49 ± 13^{a}	228 ± 32^{a}	322 ± 46^{a}	221 ± 25^{a}	198 ± 19^{a}
	Treatment 2	52 ± 11^{a}	238 ± 26^{a}	353 ± 42^{a}	235 ± 29^{a}	217 ± 22^{a}
B. rotundiformis	Treatment 1	-	-	1082 ± 129^{b}	1426 ± 101^{b}	2070 ± 224^{b}
	Treatment 2	-	-	1124 ± 96^{b}	1506 ± 86^{b}	2145 ± 193^{b}
Both rotifers combined	Treatment 1	49 ± 13^{a}	228 ± 32^{a}	1404 ± 117^{b}	1646 ± 89^{b}	2268 ± 233^{b}
	Treatment 2	52 ± 11^{a}	238 ± 26^{a}	1477 ± 133^{b}	1741 ± 107^{b}	2363 ± 192^{b}

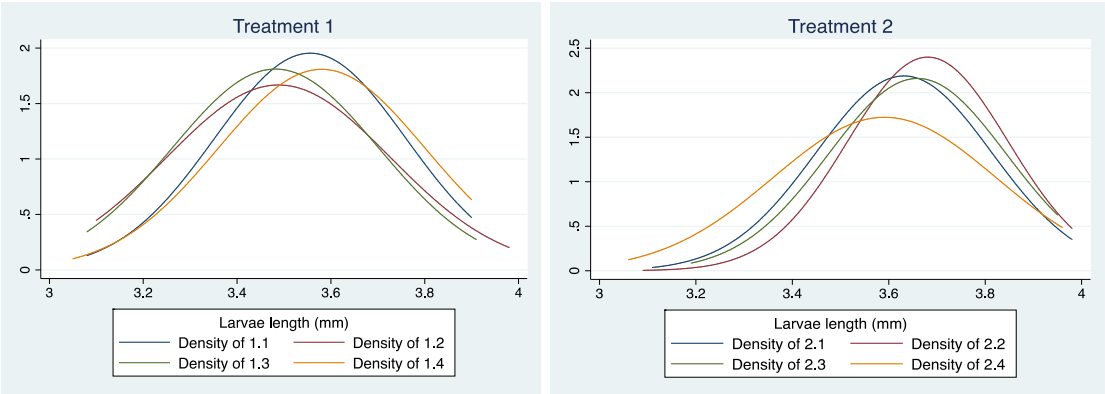

Figure 4.3. The larval total length distributions at 240 hph from four replicates in two treatments. Treatment 1: larvae fed non-enriched rotifers: *P. similis* (0.5 – 1 mg DW.L^{-1}.day^{-1} after and before *B. rotundiformis*, respectively) + *B. rotundiformis* (0.5 mg DW.L^{-1}.day^{-1}). Treatment 2: Same as treatment 1, but rotifers were probiotic-enriched prior to feeding to larvae.

4.3.4. Experiment 4: Effect of different rearing protocols to the performance and survival of the larvae from day 10 to 25

Feeding incidence and percentage: The first feeding incidence of the larvae on *Artemia* and compound diet was noted on day 11 (Table 4.13). The % of feeding larvae on *Artemia* in 4 treatments from day 10 to 15 was noted in Table 4.14. On day 11, there were no significant differences in the % of feeding larvae on *Artemia* (from 22 to 33%) (one-way ANOVA, Tukey-Kramer, $p>0.05$). On day 12, the % of feeding larvae on *Artemia* was significantly different among treatments (one-way ANOVA, $p<0.001$). On day 12, *Artemia* were found in all the larval gut content from treatment 3 (*Artemia* + compound diet), which is significantly higher than those from treatment 1 (*Artemia* + rotifers, 67%) or treatment 4 (*Artemia* + rotifers + compound diet, 56%) (Tukey Kramer, $p<0.05$). On day 11, the larvae started to eat compound diet. At this day, there were no significant differences on the % of feeding larvae on compound diet, ranging from 11 to 22% (one-way ANOVA, $p>0.05$). On day 12, the % of feeding larvae on compound diet was significantly different among treatments (one-way ANOVA, $p<0.001$). On day 12, 100% of the larvae at treatment 3 (*Artemia* + compound diet) and treatment 5 (*Artemia* + rotifers + compound diet) was found with compound diet in the gut, which is significantly higher than those from treatment 2 (compound diet + rotifers, 78%) (Tukey-Kramer, $p<0.05$). On day 13, 100% larvae from treatment 2, 3 and 4 were found with compound diet in the gut (Table 4.14).

Survival on day 25: On day 25, the survival was significantly different among treatments (Figure 4.4, one-way ANOVA, $p<0.001$). The survival in treatment 1 (100%

live food, *Artemia* + rotifers, 94%) was 12 – 114% higher than any other treatment (Tukey-Kramer, $p<0.05$). It was followed by treatment 4 (*Artemia* + rotifers + compound diet, 84%) (Tukey-Kramer, $p>0.05$). The lowest survival was noted in treatment 3 (*Artemia* + compound diet, 44%) (Tukey-Kramer, $p<0.05$).

Larval total length on day 25: On day 25, the larval length (mean ± S.D.) was significantly different among treatments (Kruskal-Wallis, $p<0.031$, n=3, different sample size) (Table 4.15). There were no significant differences in the larval length of all treatments (treatment 1: *Artemia* + rotifers, 16.6 mm; treatment 2: compound diet + rotifers, 14.6 mm; treatment 3: *Artemia* + compound diet, 16.3 mm; treatment 4: *Artemia* + rotifers + compound diet, 16.3 mm) (Kruskal-Wallis, $p>0.05$), except between those from treatment 1 and 2 (Kruskal-Wallis, $p<0.05$) (Table 4.15). The larval length in treatments 1, 2 and 4 had a normal distribution, except for those observed in treatment 3, which was right-skewed (SC = 0.55) (Skewness/Kurtosis). The result of the curve analysis showed no significant differences in the standard deviations of larval total length among treatments (F-Fisher, $p>0.05$) (Figure 4.5).

Table 4.13. Start day of feeding on *Artemia* instar I and compound diet.

Treatments	Feeding incidence on *Artemia*	Feeding incidence on compound diet
Treatment 1	Day 11	-
Treatment 2	-	Day 11
Treatment 3	Day 11	Day 11
Treatment 4	Day 11	Day 11

Table 4.14. The % of feeding larvae (mean ± S.D.) on *Artemia* instar I and compound diet from day 10 to 13 in all treatments (one-way ANOVA, Tukey-Kramer for multiple comparisons, different letters from each column indicate significant differences in the percentage of feeding larvae among treatments on different sampling points, n=3)

Treatments	Day 10	Day 11	Day 12	Day 13
	The percentage of larvae feeding on *Artemia* (%)			
Treatment 1	0 ± 0^{a}	22 ± 6^{b}	67 ± 0^{b}	100 ± 0^{c}
Treatment 2	-	-	-	-
Treatment 3	0 ± 0^{a}	33 ± 10^{b}	100 ± 0^{c}	100 ± 0^{c}
Treatment 4	0 ± 0^{a}	22 ± 6^{b}	56 ± 6^{b}	100 ± 0^{c}
	The percentage of larvae feeding on compound diet (%)			
Treatment 1	-	-	-	-
Treatment 2	0 ± 0^{a}	22 ± 6^{a}	78 ± 6^{a}	100 ± 0^{b}
Treatment 3	0 ± 0^{a}	11 ± 6^{a}	100 ± 0^{b}	100 ± 0^{b}
Treatment 4	0 ± 0^{a}	22 ± 6^{a}	100 ± 0^{b}	100 ± 0^{c}

Treatment 1: fed *Artemia* + rotifers (1g DW.tank^{-1}.day^{-1} each). Treatment 2: fed compound diet + rotifers (1g DW.tank^{-1}.day^{-1} each). Treatment 3: fed *Artemia* + compound diet (1g DW.tank^{-1}.day^{-1} each). Treatment 4: fed *Artemia* + rotifers + compound diet (2/3g DW.tank^{-1}.day^{-1} each).

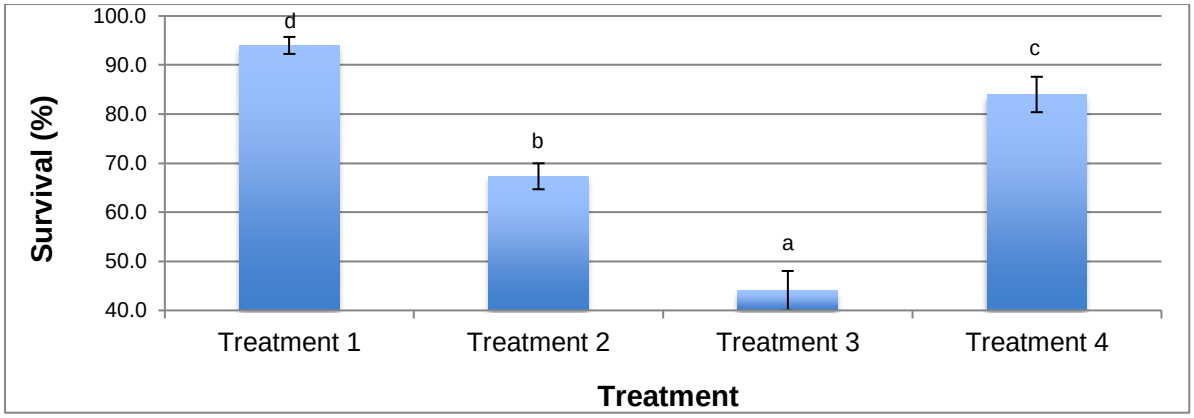

Figure 4.4. Survival (%, mean ± S.D.) on day 25 in all treatments (one-way ANOVA, Tukey-Kramer for multiple comparisons, different letters between columns indicate significant differences in the survival among treatments on day 25, n=3).

Table 4.15. Average larval length (mm, mean ± S.D., n=3) at day 10 in all treatments. Different letters in the same row indicate significant differences in the larval length at day 10 between treatments.

Treatments	**Treatment 1**	**Treatment 2**	**Treatment 3**	**Treatment 4**
Larval length (mm)	16.6 ± 1.5^{a}	14.6 ± 2.0^{b}	16.3 ± 1.5^{ab}	16.3 ± 0.9^{ab}

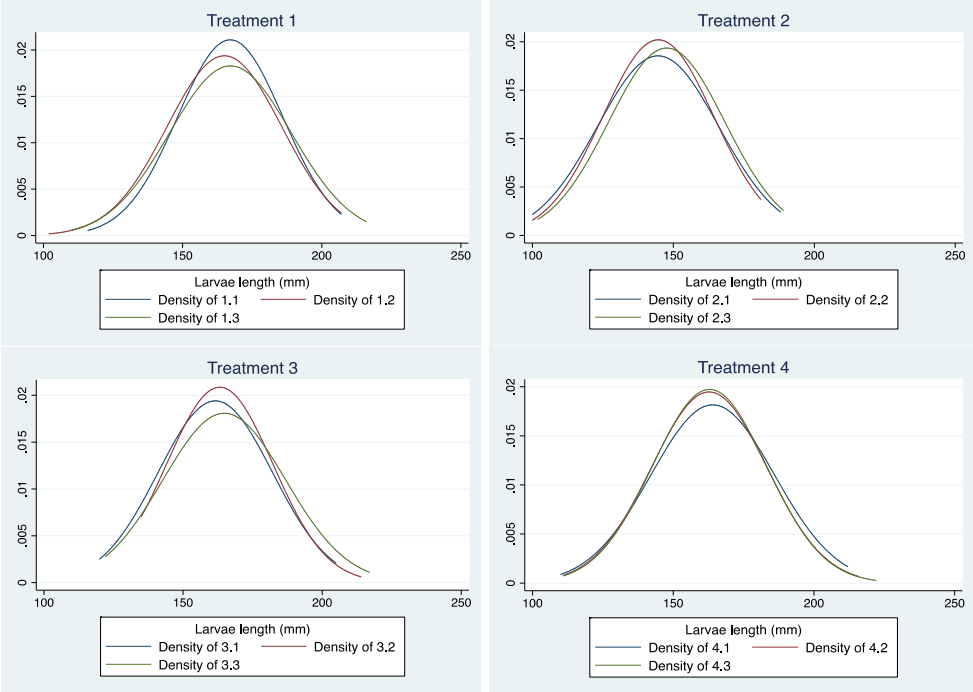

Figure 4.5. The larval length distributions on day 25 in 3 replicates in 4 treatments.

Treatment 1: fed *Artemia* + rotifers (1g DW.tank^{-1}.day^{-1} each). Treatment 2: fed compound diet + rotifers (1g DW.tank^{-1}.day^{-1} each). Treatment 3: fed *Artemia* + compound diet (1g DW.tank^{-1}.day^{-1} each). Treatment 4: fed *Artemia* + rotifers + compound diet (2/3g DW.tank^{-1}.day^{-1} each).

4.4. Discussion

It is difficult to rear the rabbit fish larvae in the period from hatching up to 6 days because of several reasons (Hara *et al.*, 1986a; Duray, 1998b). Firstly, the length of newly hatched larvae are approximate 1.50 mm (Hara *et al.*, 1986a), which is smaller

than other species, such as seabass (1.72 mm) and milkfish (3.46 mm) (Bagarinao, 1986). Furthermore, the yolk sac at hatching (0.0251 µL) is smaller than that of seabass larvae (0.0859 µL) and milkfish (0.4744 µL) (Bagarinao, 1986). After 41 – 57 hph, the yolk sac of *S. javus* larvae was completely absorbed. Furthermore, the rabbit fish larvae were very sensitive to stress and handling, which resulted in a low survival during the early stages (within 6 days after hatching) (Hara *et al.*, 1986a; Ayson, 1989; Diani *et al.*, 1990; Duray, 1998b). Hara *et al.* (1986b) and Ayson (1989) reported that having a food small enough to be ingested is indispensable for larval rabbit fish before day 6.

In this study, *P. similis* and *B. rotundiformis* were tested as initial food for larvae in the first experiment. The feeding incidence was earlier and significantly higher survival was obtained in the treatments fed *P. similis,* than those fed *B. rotundiformis.* This is due to the size difference between the two rotifers. The body size, length and width, of *P. similis* were 38.1% smaller and 60.3% narrower than *B. rotundiformis* (Wullur *et al.*, 2009). The DW of *B. rotundiformis* was 5x higher than *P. similis*. Although the larvae ingested more food (in DW) when they fed on *B. rotundiformis*, their survival was low due to the delay in start feeding. In addition, the yolk-sac of rabbit fish larvae is exhausted at 48 – 72 hph (Bryan and Madraisau, 1977; Juario *et al.*, 1985; Bagarinao, 1986; Hara *et al.*, 1986b; Kohno *et al.*, 1988; Duray, 1998b). Kohno *et al.* (1986) found that at 55.5 hph, when the *S. guttatus* mouth had a size of 200 µm, the rabbitfish larvae could start feeding on exogenous food. At this stage, the *B. rotundiformis* is still too big (Kohno *et al.*, 1986; Watanabe and Kiron, 1994). This was confirmed by the results from experiment 1, where the feeding incidence of larvae on *B. rotundiformis* occurred at 60 hph. This causes big problems for rearing fry of rabbit fish. Some studies suggested mixed species of plankton, fertilized oyster eggs or oyster D-larvae for initial feeding, but this resulted in poor or inconsistent survival (May *et al.*, 1974; Popper and Gundermann, 1976; Popper *et al.*, 1976; Kitajima *et al.*, 1980). In previous studies, a smaller fraction of *Brachionus sp.* was obtained either by selecting small-sized individuals through screening, or isolating and culturing small-sized rotifers (Fukusho and Iwamoto, 1980; 1981; Fukusho and Okauchi, 1982; Tsukashima *et al.*, 1983). Especially the study of Tsukashima *et al.* (1983) obtained good results in siganid larval rearing using a combination of both above methods, while Kitajima et al. (1980) obtained mass mortality when using large *Brachionus* rotifers (280 µm). Rotifer

B. rotundiformis were still considered to be one of the most suitable food for siganid larvae, until the rotifer *P. similis* was discovered.

In this study, the first feeding incidence and survival indicated that *P. similis* is by far the better starter food for rabbit fish larvae than the SS-type *B. rotundiformis.* The studies of Wullur *et al.* (2009; 2011); Hirai *et al.* (2013); Wullur *et al.* (2013); Hagiwara *et al.* (2014) also concluded that *P. similis* has been proven to be one of the most suitable live foods for the first feeding stage of a variety of marine species with a very small mouth, *e.g.* seven-band grouper *E. septemfasciatus*, the rusty angelfish *C. ferrugata*, the humphead wrasse *C. undulatus* and special oesophagus, *e.g.* Japanese eel *A. japonica*. The importance of *P. similis* after the first feeding period (from day 5 or 6 onwards) has been questioned. In fact, due to the difference in DW, the fish larvae ingest more DW by feeding on *B. rotundiformis* than *P. similis*. Thus, the second experiment of this study was carried out in order to investigate the importance of *P. similis* as the only food, in comparison to co-feeding with the *B. rotundiformis* (the latter from day 5 onwards or later). The amount of rotifers in the gut and the survival of the fish larvae in control 2 of experiment 2 confirmed the results of experiment 1. The results from this experiment showed the importance of the *B. rotundiformis* for the survival of rabbit fish larvae when introduced into the culture system from day 6 onwards. Indeed, the introduction of *B. rotundiformis* at day 6 resulted in the best survival of all treatments. From the moment *B. rotundiformis* was introduced in the tank, while the amount of ingested *P. similis* remained constant, the number of *B. rotundiformis* increased with time. Related to the size of the food, Hara *et al.* (1986b), Kohno *et al.* (1988), Ayson (1989) reported that size of the food has a great impact on the growth and survival of the larvae before day 6. Up to day 6 after the larvae had hatched, most rotifer strains, even the *B. rotundiformis* are still too big for the mouth of the larvae (200 µm at first feeding), and after day 6, the *B. rotundiformis* was more suitable than *P. similis* for the feeding of siganid larvae. In addition, the *B. rotundiformis* is heavier in DW than *P. similis* (Wullur et al., 2011), therefore, feeding predominantly on a bigger prey could clearly help fish larvae to increase the energy-per-catch of prey, fostering improved survival and growth (Lotrich, 1973; Kitajima *et al.*, 1979; Tanaka *et al.*, 2005; Akazawa *et al.*, 2008; Wullur *et al.*, 2009; 2011; Wullur *et al.*, 2013). Previous studies indicated that size, motion and color are important factors for the food selection of larvae (Utne - Palm, 1999; Shaw *et al.*, 2003; Tanaka

et al., 2005; Akazawa *et al.*, 2008). Wullur *et al.* (2011) observed that the swimming behavior and color of these two rotifers were similar; therefore, the preference of rabbit fish larvae to *P. similis* before day 6 was likely related to the smaller body size. The change of preference by the rabbit fish larvae to a bigger rotifer (*B. rotundiformis*) from day 6 onwards could improve feeding efficiency and survival of the larvae. In this study, despite the biomass of *P. similis* ingested by the fish larvae being always lower than the biomass of *B. rotundiformis,* performance of rabbit fish larvae in terms of survival in the former case, was always better There is a possible effect of prey body size on prey digestibility by fish larvae. Smaller prey provides larger surface to volume ratio, probably making it more accessible to digestive enzymes. The digestion might even be more enhanced by the fact that small amount of ingested feed tend to stay longer in the digestive tract (Tseitlin, 1980).

After the most appropriate co-feeding scheme was found, the experiment on the effects of the addition of probiotic-enriched rotifers on the larval rearing was performed. The amount of rotifers in the larval gut in treatment 1 of this experiment confirmed results of experiment 2. The addition of probionts to the rotifer culture did not have any effect on the ingestion of rotifers, however, the results of this test showed that the larvae fed probiotic-enriched rotifers had significantly higher survival than the larvae fed non-enriched rotifers. The feeding of probionts-enriched rotifers to the larvae did lead to bigger larvae, which had a more homogenous size, and more right-skewed distribution than those in treatment 1 (without probionts-enriched rotifers). The effects of the addition of *L. plantarum* and *L. helveticus* and *L. lactis* to the rotifer culture prior to feeding to turbot larvae were tested by Gatesoupe (1991b) and Harzevili *et al.* (1998a). The addition of *L. plantarum* and *L. helveticus* to the rotifer culture improved the survival and mean weight of turbot larvae, reduce aerobic bacterial loads and inhibit the growth of *V. anguillarum* in the water. The MC of rotifers plays an important role to shape the larval gut MC, and thus deliver a direct effect to the performance of fish larvae (Dawood *et al.*, 2016; Le *et al.*, 2017). Attramadal *et al.,* 2012a, 2012b confirmed that the bacteria composition associated with live food affects the gut MC of fish larvae. Hence, the manipulation of the MC in live food culture (*e.g.* bioencapsulation) could be beneficial to the fish larvae. In fact, the application of bioencapsulation of bacteria via rotifers or *Artemia* to deliver LAB and *B. subtilis* to the gut of fish larvae has been reported by Makridis *et al.* (2000) and Vadstein *et al.,* (2013). The presence of probiotics in the larval gut could increase digestive enzyme

excretion; improve immunostimulation, enhance production of antagonistic compounds and surpress the growth of pathogens (De Schryver *et al.,* 2012). In conclusion, the bioencapsulation of LAB and *B. subtilis* should be applied in the rabbit fish larviculture to increase the survival and improve the performance of the larvae, although the mode of action in this particular case remains to be established.

In this study, the effect of different rearing protocols on the performance and survival of larvae from day 10 to 25 has also been tested. The results of this experiment showed that rotifers still play an important role as a food source to the fish larvae beyond day 10. In treatment 3, when rotifers were not available, the fish larvae did not eat anything at 240 hph, resulting in the lowest survival compared to other treatments. In this study, the size of rotifer *B. plicatilis* (230 µm) was smaller than *Artemia* instar I (500-600 µm) and compound diet (400 – 600 µm) (Hagiwara *et al.*, 1995; Lavens and Sorgeloos, 1996). At 240 hph, the larvae were still unable to feed on *Artemia* instar I or compound diet. This is due to the size and the ability of the larvae to recognize the *Artemia* instar I and compound diet as food. Therefore, it would take 1-2 days for the larvae to be able to feed on a new food (weaning period). In conclusion, without rotifers in the feeding scheme (co-fed *Artemia* instar I and compound diets), the survival of the fish larvae was significantly lower than those in other treatments with the addition of rotifers. The importance of rotifers after day 10 was also proven by Juario *et al.* (1985); Bagarinao (1986); Ordonio-Aguilar (1995); Pechmanee (1997); Duray (1998b); Rachmansyah *et al.* (2007); Moorhead and Zeng (2017) where rotifers were still co-fed to the fish larvae until day 17 to 20 in various species of fish, including siganids. After that, *Artemia* appeared to be the most suitable for the larvae. Compound diet was good for larvae when combined with rotifers and *Artemia* instar I (Vinh Chau, Vietnam). The feeding schemes including *Artemia* instar I and rotifers resulted in significantly higher survival and total length of larvae than the feeding scheme without *Artemia* instar I. Furthermore, the treatment in which the larvae were co-fed *Artemia* instar I and rotifers gave the best survival (94%). However, since *Artemia* is more expensive compared to the compound diets, the treatment in which *Artemia* instar I, rotifers and compound diets are co-fed, which still ended up with 84% survival, would be important to be considered from a commercial point of view in the larval rearing. The role of *Artemia* in the co-fed schemes with compound diets in larval rearing of marine fishes, was reported by Juario *et al.* (1985); Bagarinao (1986); Hara

et al. (1986a); Hara *et al.* (1986b); Hara *et al.* (1986c); Ayson (1989); Ordonio-Aguilar (1995); Quinitio and Duray (1996); Pechmanee (1997); Rosenlund *et al.* (1997); Duray (1998b); Canavate and Fernández-Dıaz (1999); Sugama *et al.* (2001); Marte (2003); Rachmansyah *et al.* (2007); Moorhead and Zeng (2017). It was found that co-feeding in a number of commercially important species, such as European sea bass (*D. labrax*), gilthead sea bream (*S. aurata*), turbot (*S. maximus*), Senegal sole (*S. senegalensis*) and Atlantic halibut (*H. hippoglossus*), under commercial hatchery conditions, resulted in the highest survival when the live-compound diet ratio was 1:1 (Rosenlund *et al.*, 1997; Canavate and Fernández-Dıaz, 1999). On day 25, there was no difference in the body length distribution. However, the average larval total length was significantly higher in larvae fed *Artemia* instar I + rotifers than in those fed compound diet + rotifers. It showed that *Artemia* instar I in the diet would improve the food efficiency ratio and thus, increases the larvae size compared to the compound diets. In fact, *Artemia* ingested by the fish larvae transfer their digestive enzymes to the fish larvae (by autolysis or as zymogens), thus, improving the larval endogenous digestive system (Cahu and Infante, 2001; Kolkovski, 2001). Moreover, the length of larvae fed *Artemia* + compound diet had a right-skewed distribution, while those from other treatments were normal. Without the rotifers in the diet, feeding opportunities decreased for smaller larvae, which enabled a hierarchic structure to emerge. Once the hierarchic structure occurs, differential growth begins, and the variability in growth between larger and smaller individuals increases with time as larger fish inhibit the feeding activity of smaller fish, resulting in mortalities of smaller larvae due to starvation and cannibalism. Hence, the larval length distribution skewed to the right (Gershanovich, 1983; Onders *et al.*, 2008; 2011). Therefore, larvae should be graded at this stage (day 25) to reduce the heterogeneity in the larval length, which might lead to the cannibalism among larvae.

In conclusion, the co-feeding of probiotic-enriched *P. similis* and *B. rotundiformis* (with the ratio 1:1), where *B. rotundiformis* was added from day 6, resulted in the highest survival of fish larvae at 240 hph. After that, co-feeding of *Artemia*-rotifers (1:1) or *Artemia*-rotifers-compound diets (1:1:1) in the fish larval rearing from day 10 to day 25 resulted in the best survival during the larval rearing.

The current protocol did not include LC-PUFA enrichment of the live food, a common practice in marine fish larviculture (see table 4.1). It was hypothesized that data on the

PUFA composition of wild larvae entering into lagoons would provide information on the natural PUFA content of wild larvae and hence provide further information on the nutritional requirements of rabbit fish. In addition the larviculture protocol of rabbit fish as developed above includes a consistent application of probionts, with apparent successful results. It was anticipated that the microbial community composition (MCC) in hatchery larvae would be considerably different from larvae caught in the wild. Knowledge on the composition of microbial communities in wild rabbit fish larvae might also provide information on how to further optimise larval rearing protocols. Hence in the next chapter PUFA content and MCC in wild larvae were analysed and compared to those from hatchery reared larvae as obtained by the current protocol.

CHAPTER 5

PUFA composition and gut microbial community analysis of hatchery-reared larvae and wild larvae of rabbit fish (*Siganus guttatus*) from three locations over a three-year period

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Abstract

In this study, the PUFA composition of body tissue and the gut MC of hatchery and wild larvae of rabbit fish at 3 different locations in Vietnam (ThuanAn, QuangNam and BinhDinh) over a period of 3 years were investigated. Results from this study showed that rabbit fish larvae contained high proportion of ARA. The ARA proportion of wild larvae was stable between sampling locations over the three-year period, and no significant differences were detected in the ARA level between wild and hatchery samples. The DHA/EPA ratios in fish larvae were very high and varied between locations. The PUFA composition is within the normal range in the wild. The difference between temperatures at the 3 locations are negatively correlated with the DHA content and the DHA/EPA ratio, but not the EPA content. The results of Illumina analysis of 16S libraries of wild samples showed that the location affected the gut MC composition. The bacteria that were identified in the rabbit fish gut content were mainly belonging in the Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria and Firmicutes phyla. In addition, the gut MC diversity and composition of hatchery larvae, which were fed probiotic-enriched rotifers, was completely different than those in the gut of wild larvae. The probiotic strains (*Lactobacillus plantarum, L. fermentum*, *L. brevis, Pediococcus acidilactici, P. pentosaceus and B. subtilis*) were predominant in the hatchery larvae. Next, relative genetic distances between ThuanAn and BinhDinh/QuangNam were bigger than those between BinhDinh-QuangNam. More importantly, sampling location had significant impacts on the MC. The diversity of gut MC increased significantly from the north to the south. In conclusion, the current larval rearing protocol is reliable in terms of PUFA profile and probiotics. The hatchery larvae should not be treated differently in the future.

Keywords: rabbit fish*, Siganus guttatus*, PUFA, microbial community, Illumina

5.1. Introduction

The rabbit fish (*S. guttatus*), a native species in Southeast Asia, including Central Vietnam, is an important commercial fish in this area (Juario *et al.*, 1985; Hara *et al.*, 1986c; Mien *et al.*, 2000; Ayson *et al.*, 2014). So far, the fingerlings of this species for aquaculture were only obtained from the wild. They were collected at the river mouths in the Central Vietnam, mainly in Thua Thien Hue, Quang Nam and Binh Dinh provinces. The smallest larvae size, which was observed in the river mouths at the collecting points, is 14 – 18 mm (Juario *et al.*, 1985; Hara *et al.*, 1986c; Mien *et al.*, 2000; Ayson *et al.*, 2014). Since 2015, artificial reproduction of rabbit fish has been introduced in Vietnam, and hatchery larvae have been provided to the aquaculture sector (Le *et al.*, 2017). It is essential to gain insight into the overall health of hatchery larvae, which might provide important hints to further improve the farming of larvae. Previous studies showed that long chain unsaturated fatty acids in the food play an important role in the growth performance and stress resistance of the fish larvae (Dhert *et al.*, 1990; Dhert *et al.*, 1992; Ako *et al.*, 1994a). Comprehension of the nutritional requirements for the PUFA content, the DHA/EPA ratio in marine fish larvae can contribute to the establishment of an optimal diet for larval rearing (Watanabe *et al.*, 1978a; Watanabe *et al.*, 1982).

Besides the PUFA content, the investigation of gut MC composition is also important (Romero *et al.*, 2014). The gut MC composition is affected by the interaction between host nutrition, environment and genetic factors (McFall-Ngai *et al.*, 2013). A comprehensive understanding of the gut MC is necessary to explain its function in the overall health status of fish, especially in thelarval stage (Ringø *et al.*, 2010; Bakke *et al.*, 2013; Giatsis *et al.*, 2014). In recent years, the amplicon sequencing techniques, such as Roches' 454, Illumina MiSeq, NextSeq 500 and HiSeq 2000 have provided valuable alternatives to tackle the limitations of older techniques, such as direct bacterial culturing and 16S rRNA amplification followed by DGGE, TTGE or T-RFLP in terms of *e.g.* number of bacteria identified (Romero and Navarrete, 2006; Hovda *et al.*, 2007; Navarrete *et al.*, 2009; van Kessel *et al.*, 2011; Geraylou *et al.*, 2013; Green *et al.*, 2013; Star *et al.*, 2013; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b; Zarkasi *et al.*, 2014; Zarkasi *et al.*, 2016). By using the molecular technologies in combination with the sequencing technique, the diversity and MC composition in the fish gut have been described in more details (Austin, 2006; Kim *et al.*, 2007; Namba *et al.*, 2007; Wu

et al., 2010; Lan and Love, 2012; Wu *et al.*, 2012a; Larsen *et al.*, 2013). Previous studies showed that the composition of gut MC had a crucial function in fish development, nutrient digestion, immune function and protection from invasive pathogens (Bird *et al.*, 2010; Nayak, 2010; Wong and Rawls, 2012; Engel and Moran, 2013; Viaud *et al.*, 2013; Romero *et al.*, 2014).

The aim of this study was to investigate the PUFA content and gut MC composition of wild rabbit fish larvae from three different locations (Thua Thien Hue, Quang Nam and Binh Dinh) over a 3-year period (2014 to 2016) and to compare them with the PUFA content and gut MC of hatchery larvae. This might provide further insight into the overall health status of hatchery larvae.

5.2. Materials and methods

5.2.1. Location and sampling procedure

Wild larvae were collected from 3 different river mouths in Central Vietnam. Location 1 (namely ThuanAn) was in Thua Thien Hue province (13°45'42.4"N 109°14'45.5"E). Location 2 (namely QuangNam) was in Quang Nam province (15°52'38.1"N 108°24'00.2"E). Location 3 (namely BinhDinh) was in Binh Dinh province (16°34'46.0"N 107°37'17.5"E). These larvae were collected between 8th and 10th of June in the year 2014, 2015 and 2016, when the wild larvae first appeared in the river mouths (Figure 5.1). Water parameters at sampling points (water temperature, salinity and pH) were measured at 2 meters depth using an electronic device (Horiba, Japan) at 5 different points at the sampling areas (Figure 5.1). Larvae were collected by fishing net in the morning between 7 – 8 AM, washed with nuclease free water (Promega, USA), and subsequently kept on ice and transported to the laboratory for a freeze-dried process. The freeze-dried samples were preserved at -20 °C for the FAME and gut MC analysis. The hatchery larvae were obtained from a batch of larvae fed rotifers enriched with a mixture of *L. plantarum, L. fermentum, L. brevis, P. acidilactici, P. pentosaceus* and *B. subtilis* (10^6 CFU.mL^{-1} of each species, totally 6 x 10^6 CFU.mL^{-1}) following the method described by Le *et al.* (2017). From the hatchery (25 days old larvae) and each wild sampling year + location, 10 larvae were collected for FAME analysis and 10 larvae were collected for gut MC analysis. The FAME and gut MC analysis were done on each individual (for each multivariate dataset: sample size = 1 and sample number = 10). The details on sampling locations, time, larval length and abiotic factors of both wild and hatchery larvae were noted in Table 5.1.

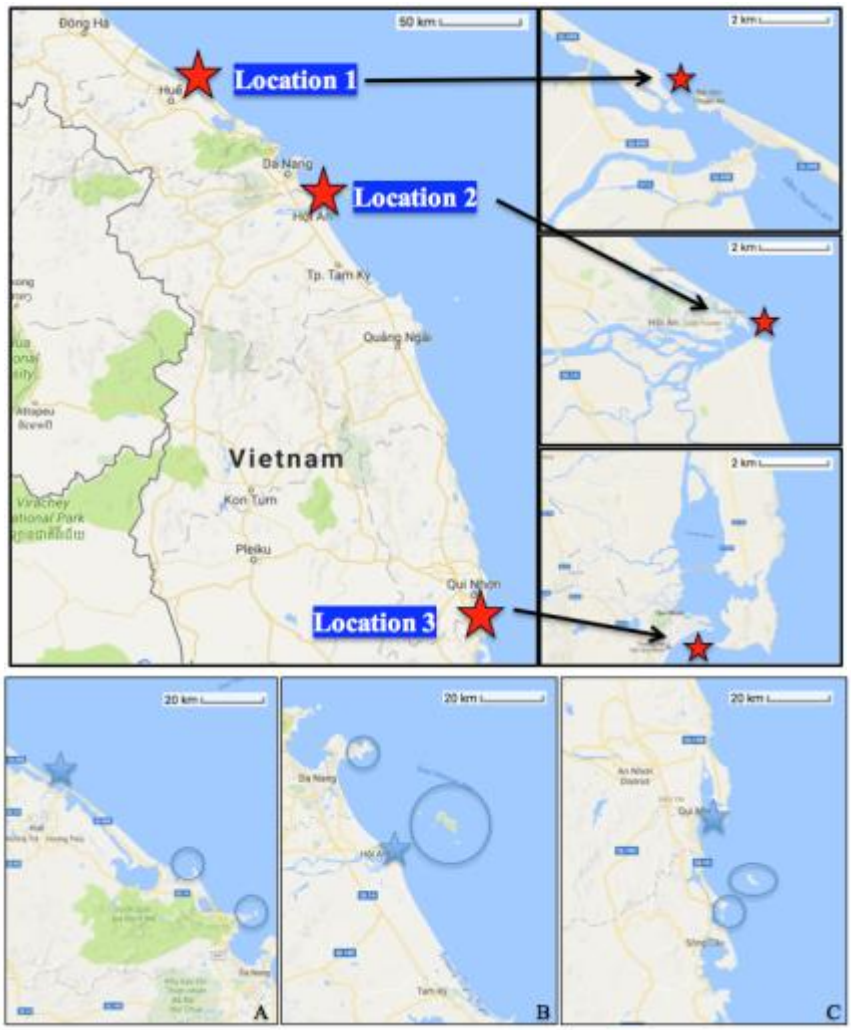

Figure 5.1. Sampling locations of wild rabbit fish over a three-year period (2014-2016). Location 1: ThuanAn (Thua Thien Hue province); location 2: QuangNam (Quang Nam province); location 3: BinhDinh (Binh Dinh province) (Source: https://www.google.com/maps/@15.34538,108.3821484,7.84z). Sampling locations (star) and coral reef breeding ground (circle) of wild rabbit fish. A: ThuanAn; B: QuangNam; C: BinhDinh (Source: A. https://www.google.be/maps/@16.3975183,107.9632426,9.7z?hl=en; B. https://www.google.be/maps/@15.9331892,108.6010506,9.7z?hl=en; C. https://www.google.be/maps/@13.7129966,109.1235103,9.7z?hl=en)

Table 5.1 Sampling location, time, larval length and abiotic factors of wild and hatchery larvae (mean ± S.D., n=10 for larval length, n=5 for abiotic factors). No significant differences were found in the average larval length between datasets (ANOVA, n=10).

No	Time	Location	Origin/Province	Larval length (mm)	Temperature (°C)	Salinity (g.L^{-1})	pH
1	June 2016	Hatchery	Hatchery-reared	17.53 ± 1.17^{a}	30.1 ± 0.1	28.2 ± 0.1	7.7 ± 0.1
2		ThuanAn	Thua Thien Hue	17.49 ± 0.87^{a}	26.8 ± 0.3	27.2 ± 0.2	7.6 ± 0.2
3	June 2014	QuangNam	Quang Nam	17.88 ± 0.79^{a}	27.4 ± 0.4	27.5 ± 0.3	7.7 ± 0.1
4		BinhDinh	Binh Dinh	17.31 ± 1.13^{a}	29.7 ± 0.3	28.2 ± 0.2	7.7 ± 0.1
5		ThuanAn	Thua Thien Hue	17.26 ± 0.74^{a}	27.1 ± 0.3	27.3 ± 0.2	7.5 ± 0.1
6	June 2015	QuangNam	Quang Nam	17.35 ± 0.91^{a}	28.6 ± 0.2	27.9 ± 0.2	7.7 ± 0.1
7		BinhDinh	Binh Dinh	17.33 ± 0.94^{a}	30.3 ± 0.3	28.4 ± 0.2	7.7 ± 0.1
8		ThuanAn	Thua Thien Hue	17.14 ± 1.02^{a}	27.0 ± 0.2	27.5 ± 0.3	7.6 ± 0.1
9	June 2016	QuangNam	Quang Nam	17.30 ± 0.95^{a}	29.3 ± 0.2	28.4 ± 0.2	7.7 ± 0.1
10		BinhDinh	Binh Dinh	17.39 ± 0.96^{a}	30.5 ± 0.4	28.5 ± 0.2	7.8 ± 0.2

5.2.2. FAME analysis

Whole fish were used for FAME analysis at the Lab. of Aquaculture & ARC, Ghent University, Belgium. The FAME analysis followed the modified procedure of Lepage and Roy (1984), was conducted as described by Coutteau *et al.* (1995). The method employs a direct acid catalyzed transesterification without prior lipid extraction using methanol/acetyl chloride (20/1). The PUFAs were extracted with hexane, redissolved in iso-octane and analyzed by on column injection in a Chrompack CP9001 chromatograph operating with hydrogen (100kPa) as carrier gas and flame ionization detection, equipped with a 2.5 m methyl deactivated precolumn connected to a 50 m polar capillary column (BPX70, SGE, Australia) (0.32 mm internal diameter, 0.25 µm layer thickness). Temperature was programmed to rise from 85°C to 182°C. Peaks were identified by comparison with reference standards (Nu-Check Prep, USA).

5.2.3. Illumina sequencing for gut MC analysis

The freeze-dried fish samples were first hydrated in sodium phosphate buffer prior to extraction. After that, the gut was removed from the fish larvae. The DNA of the gut content was extracted using the FastDNA Spin Kit for Soil (MP Biochemicals, USA), according to the manufacturer's instructions. The DNA concentration in the extract was then normalized to the final value of 1 ng/µL, and the extracts were sent to LGC Genomics (Berlin, Germany) for Illumina on the Miseq platform. The Illumina protocol was written by Kim De Paepe and corrected by Berthold Fartmann (LGC genomics, Germany). First, the bacterial 16S rRNA gene was amplified using a primers 341F CCTACGGGNGGCWGCAG (forward) and 785R GACTACHVGGGTATCTAAKCC (reverse) (Klindworth *et al.*, 2013). The PCR reaction was carried out in 20 µL volume of MyTaq buffer containing 1.5 units of MyTaq DNA polymerase (Bioline, USA) and 2 µL of BioStabII PCR Enhancer (Sigma, USA). For each DNA sample, both primers carried the same unique 10-nt barcode sequence. The PCR protocol consisted of an initial denaturation step at 96°C for 2 minutes; followed by 20 cycles at 96°C for 15 sec, 50°C for 30 sec, 70°C for 90 sec. Gel electrophoresis was carried out to determine the DNA concentration of amplicons of interest. Up to 48 samples carrying different barcodes were pooled together (20 ng DNA of each sample). To remove the primer dimer and other by-products, the pooled samples were purified with one volume AMPure XP beads (Agencourt, USA), followed by a MinElute columns (Qiagen, The Netherlands) purification step. The purified DNA (100 ng) was used to construct Illumina libraries by means of adaptor ligation, using the Ovation Rapid DR Multiplex

System 1-96 (NuGEN, USA). The libraries were pooled together, and the size of DNA fragments was determined with gel electrophoresis. An Illumina MiSeq using V3 Chemistry (Illumina) was used to finalize the sequencing. The sequencing quality was assessed by including a mock community (in triplicates) in the sequencing run. The mock community is an in-house assembled community that was pooled together from 10 distinct strains based on equal qPCR copies (De Paepe et al., 2017).

5.2.4. MC data analysis

Amplicon sequence processing: The mothur software package (1.39.5) was used to process the amplicon sequencing data on a GNU/Linux 3.16.0-46-generic x86_64 system in accordance with the guidelines of Schloss *et al.* (2009). Forward and reverse reads were assembled into contigs by a heuristic approach, taking the Phred quality scores into account. Ambiguous contigs or contigs with unsatisfying overlap were removed, and the remaining sequences were aligned to the mothur formatted silva seed v123 database. Sequences that did not align within the region that was targeted by the primer set or sequences with homopolymer stretches with a length >12 were removed. The sequences were pre-clustered, allowing 1 mismatch for every 100 bp of sequence. Chimeric sequences were removed with UCHIME (Edgar *et al.*, 2011). The sequences were classified with a naive Bayesian classifier, using the RDP 16S rRNA gene training set, v.14 with an 85% cut-off for the pseudobootstrap confidence score. Taxa annotated as unknown, Archaea, Chloroplast, Mitochondria, Eukarya at the kingdom level were excluded. Sequences were binned into operational taxonomic units (OTUs) at a 3% dissimilarity level, as identified by the preceding classification step. A table containing the abundances of the OTUs, and their taxonomic assignments was generated.

Amplicon data processing: Samples were rescaled by taking the proportions of each OTU, multiplying it with the minimum sample size, and rounding to the nearest integer according to the "common-scale" approach (McMurdie and Holmes, 2014). Rarefaction curves were generated for each sample to evaluate sampling depth sufficiency (Sanders, 1968; Hurlbert, 1971). Statistical analyses were carried out with R studio version 3.3.1 (R Development Core Team, 2013). Community analysis was carried out using the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen *et al.*, 2016) packages. The MC composition of the biological replicates of 3 locations and over 3 years was statistically compared with analysis of variance (ANOVA, *aov*

function) to validate that the community showed no significant difference between biological replicates. Heat maps were generated on different phylogenetic levels (phylum and class), using the weighted average values of the biological replicates (heat map package). The interactions between the year and location factors in the order-based Hill's numbers (Hill, 1973) were determined with two-way ANOVA (ANOVA function of the car package, v2.1-5, on a lm object with interaction). As no interaction between the year and location factors in Hill's numbers order 1st and 2nd (H1 and H2) was found, the significant differences in the H1 and H2 between different locations and years were determined with one-way ANOVA. There was interaction between the year and location factors in Hill's numbers order 0 (H0) between different locations and years. However, there was no systematic trend in the interactive effect, occluding a biological interpretation. For convenience, the significant differences in the H0 between different locations and years were further determined with one-way ANOVA. The Hill order 0 (H0) represented the OTU richness in the gut MC of larvae. The Hill order 1 (H1, the exponential of Shannon's entropy index) represented the observed OTUs numbers with the exclusion of rare species in the gut MC of larvae. The Hill order 2 (H2, the inverse of Simpson's concentration index) represented the abundance only of the more plentiful OTUs in a sample, and can therefore be regarded as a measure of "dominant OTUs concentration" (Whittaker, 1965; Hill, 1973). Non-metric multidimensional scaling (NMDS) plots were generated based on the Bray-Curtis (BC) distance measure. Significant differences in MC composition between different locations and years were determined *via* pair-wise Permutational ANOVA (PERMANOVA) with Bonferroni correction (*adonis* function, vegan package).

Relative distance of MC 3 locations and its relation to temperature: Relative distances between the MC centroids of each location were determined based on the NMDS data and compared with geographical distance. The PERMANOVA was used to evaluate the effect of temperature on the DHA and EPA content, DHA/EPA ratio and gut MC at 3 locations using the *adonis* function (vegan). The Spearman's rank order correlation (rho) was used to determine significant correlations between temperature and the DHA and EPA content, DHA/EPA ratio and gut MC.

Data deposition: The raw fastq files that were used to create the OTU table and used as a basis for the MC analysis in this paper, have been deposited in the European Nucleotide Archive (ENA) database (accession numbers PRJEB21048).

5.2.5. LC-PUFA data analysis

Significant differences in DHA/EPA ratios between groups were analyzed using one-way ANOVA, followed by Tukey-Kramer test for multiple comparisons. Differences were considered significant at $p < 0.05$. All tests were performed using SPSS 22.0.

5.3. Results

5.3.1. PUFA compositions and its relation with water temperature

There were no significant differences in the ARA levels (2.0 – 4.2%) between wild and hatchery larvae (Tukey-Kramer, $p>0.05$), except for QuangNam samples (Tukey-Kramer, $p<0.05$). The DHA/EPA ratio QuangNam and BinhDinh samples decreased from 2014 to 2016 (8.1 in 2014 to 5.6 in 2016 and 4.0 in 2014 to 2.9 in 2016, respectively) (Tukey-Kramer, $p<0.05$), but it was stable in ThuanAn (4.1 in 2016 and 4.6 in 2014) (Tukey-Kramer, $p>0.05$). The DHA/EPA ratio of hatchery larvae was 5.4, which was within the range of the wild sample (2.9 – 8.1) (Table 5.2). Difference between temperatures at 3 locations was negatively correlated with DHA content (rho=-0.6658) and DHA/EPA ratio (rho=-0.4397), but not EPA content (rho=-0.0568) of fish larvae (Table 5.3).

Table 5.2. PUFAs (composition in percentage, mean ± SD) of the hatchery and wild larvae from 3 locations over 3 years. Different letters on the same rows indicate significant differences in PUFA content between locations/hatchery for the DHA/EPA ratio (Tukey-Kramer test, n = 10).

PUFAs	ThuanAn			QuangNam			BinhDinh			Hatchery
	2014	2015	2016	2014	2015	2016	2014	2015	2016	2016
ARA	2.3 ± 0.2	2.8 ± 0.6	2.0 ± 0.7	4.2 ± 0.8	4.1 ± 0.6	4.0 ± 0.4	3.7 ± 0.4	3.4 ± 0.3	3.4 ± 0.3	3.1 ± 0.5
EPA	2.6 ± 0.4	2.5 ± 0.4	2.4 ± 0.5	1.9 ± 0.2	2.1 ± 0.4	1.7 ± 0.2	2.3 ± 0.3	2.6 ± 0.8	2.5 ± 0.6	1.7 ± 0.2
DHA	11.8 ± 1.1	10.8 ± 0.8	9.7 ± 1.6	15.3 ± 1.5	14.9 ± 2.3	9.8 ± 1.6	9.0 ± 1.5	7.2 ± 1.1	6.8 ± 0.7	8.8 ± 0.4
DHA/EPA	4.6 ± 0.4^{a}	4.5 ± 0.8^{a}	4.1 ± 0.8^{a}	8.1 ± 1.0^{b}	7.3 ± 0.9^{b}	5.6 ± 1.3^{c}	4.0 ± 0.9^{a}	3.1 ± 1.0^{d}	2.9 ± 0.9^{e}	5.1 ± 0.4^{cf}

Table 5.3. The correlation between temperature and PUFAs in wild larvae

No.	Factors	p-value (PERMANOVA)	Spearman's rho
1	Temperature and DHA	<0.0001	-0.6658
2	Temperature and EPA	0.5946	-0.0568
3	Temperature and DHA/EPA ratio	<0.0001	-0.4397

5.3.2. Gut MC of hatchery and wild larvae in 3 locations over 3 years

Diversity measurements of the hatchery and wild larval gut MC: There were significant differences in the Hill order 0, 1, 2 of hatchery and wild larval gut MC from 3 locations (ANOVA, $p<0.0001$) (Figure 5.2). The H0, H1 and H2 of the hatchery larval gut MC were significantly lower than those from wild (ANOVA, $p<0.001$).

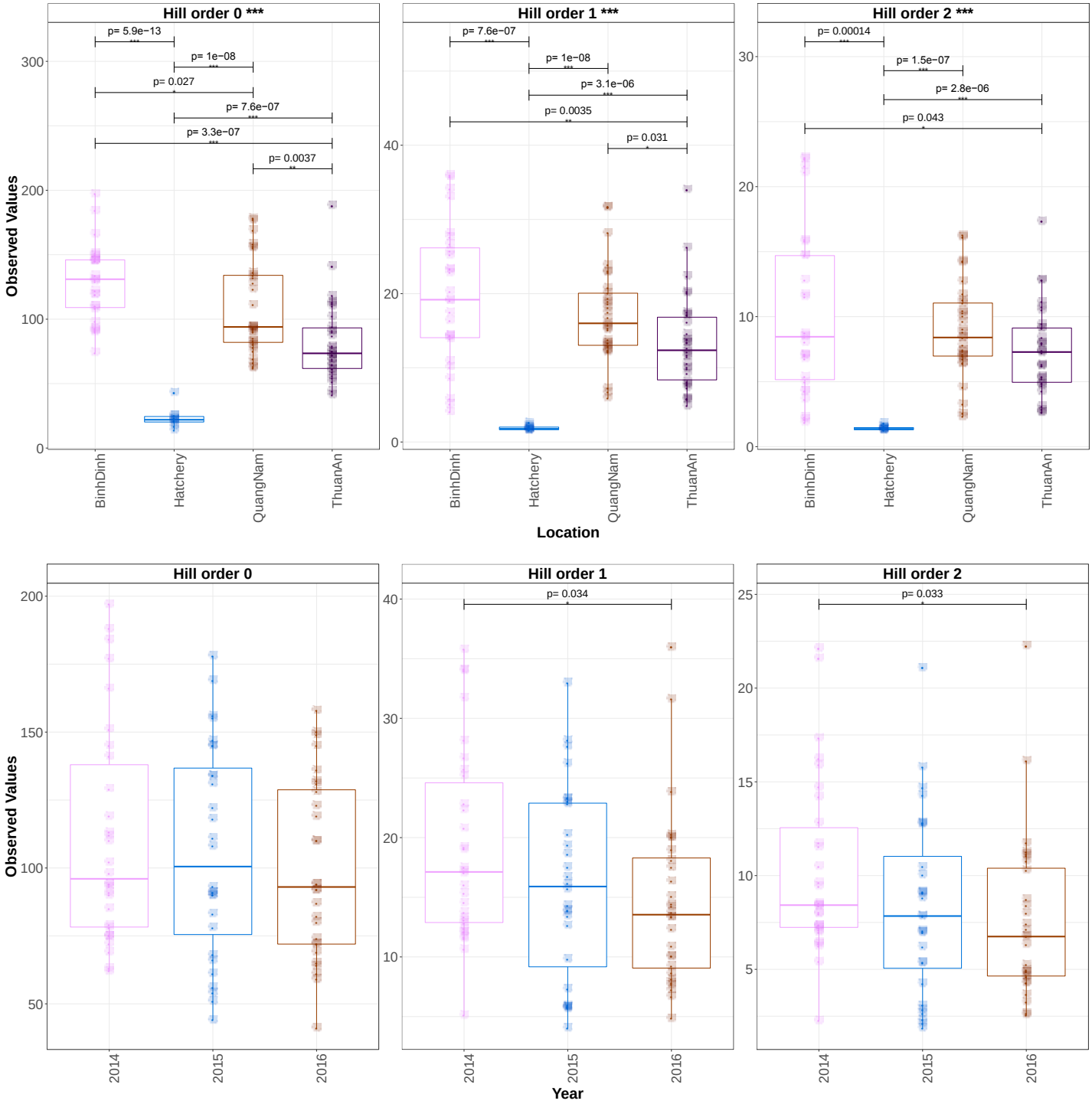

Figure 5.2. Hill numbers of the gut MC of the hatchery (Hatchery) and wild larvae from 3 locations (ThuanAn, QuangNam, BinhDinh) over 3 years. Upper figure: comparison between wild larvae from 3 locations and between wild and hatchery larvae. Lower figure: comparison between wild samples over 3 years (pooled sample over locations and years, ANOVA; n=30 for wild and n=10 for hatchery larvae).

In the wild, the H0 increased significantly from the north to south ($p<0.05$). The highest H0 was observed in BinhDinh larvae (128.7 OTUs), which was 19% higher than those from QuangNam (108.6 OTUs), 57% higher than those ThuanAn (82.1 OTUs) ($p<0.05$) and 455% higher than those from the hatchery larvae (23.2 OTUs) ($p<0.05$).

Similarly, the H1 in the gut MC from the BinhDinh was 19.8 OTUs, which was 16, 49 and 904% higher than those from QuangNam (17.1 OTUs), ThuanAn (13.3 OTUs) and hatchery (1.9 OTUs) ($p<0.05$), respectively. The highest H2 in the gut MC was in BinhDinh (10.2 OTUs) and QuangNam (8.9 OTUs) ($p>0.05$) (15 – 38% and 536 – 629% higher than those from ThuanAn (7.4 OTUs) and hatchery (1.4 OTUs) ($p<0.05$), respectively. There were significant differences in the H0 of the gut MC between years (2014: 111, 2015: 106, 2016: 100 OTUs) (ANOVA, $p<0.05$). The H1 and H2 of the gut MC in 2014 (H1: 19; H2: 10 OTUs) were significantly higher than those in 2016 (H1: 15; H2: 8 OTUs) ($p<0.05$). There were no significant differences in the H1 and H2 between the gut MC in 2015 (H1: 16; H2: 8 OTUs) and those in 2014 and 2016.

Phylogeny of gut MC of hatchery and wild larvae over a 3-year period: The Firmicutes were the most predominant phylum in the gut MC of wild (35 – 61%) and hatchery larvae (99%), except for ThuanAn in 2016, which was dominated by the Actinobacteria (35%) and Proteobacteria (34%). Other dominant OTUs belonged to the phylum Verrucomicrobia (< 22.3%), and Bacterioides (< 16.2%) (Figure 5.3). The Clostridia were the most predominant class in the gut MC in all natural locations (10 – 39%), except for the ThuanAn in 2016, which was dominated by the Actinobacteria (34%) and Alphaproteobacteria (26%). Other OTUs belonged to the classes Deltaproteobacteria (7 – 17%), Verrucomicrobiae (10 – 21%) and Erysepelotrichia (8 – 18%). The detectable classes were Betaproteobacteria (<4%), Fusobacteria (<4%), Bacteroidia (<4%), Gammaproteobacteria (<3%), Spirochaetia (<3%), Epsilonproteobacteria (<2%), Flavobacteria (<1%). The Bacilli was the most predominant class in the hatchery larval gut (98%) (Figure 5.4). Only OTUs present at an average relative abundance ≥0.1% were considered for analysis. The OTUs from Clostridiales (from 27 to 52%) was predominant in the gut of wild larvae, while OTUs from Pediococcus (83%) and Lactobacillus (15%) were predominant in the gut of hatchery larvae. The less predominant OTUs in the wild larval gut were from Akkermansia (<20%), Lachnospiraceae (<12%), Ruminococcaceae (<10%), Firmicutes (<10%), Bacteria (<10%), Bacteroidales (<7%). The Norcadia OTUs were high in larval gut from ThuanAn (10 – 24%) and QuangNam in 2014 (14%), but they were undetectable in all remaining samples. The Vibrio OTUs were 0.1 – 1.1% in the wild larvae, and were undetectable in the hatchery larval gut (Figure 5.5).

NMDS analysis of MC in hatchery and wild larvae in 3 locations in 3 years: There were no significant differences in the MC of the wild larvae over 3 years ($p>0.05$)

(Figure 5.6A). Significant differences in community composition of the larval gut MC were detected between the 3 locations in the wild (p=0.0001), and between the hatchery and 3 locations in the wild (p=0.0001) (Figure 5.6B).

Relative distance of MC from three locations and its relation to temperature: The relative genetic distances between 3 locations over 3 years and the effect of location on the MC were shown in Table 5.4. The distance between BinhDinh-ThuanAn was 1.07, QuangNam-ThuanAn was 0.68 and BinhDinh-QuangNam was 0.42. Difference between temperatures at the 3 locations were positively correlated with the genetic differences between MCs (rho=0.941).

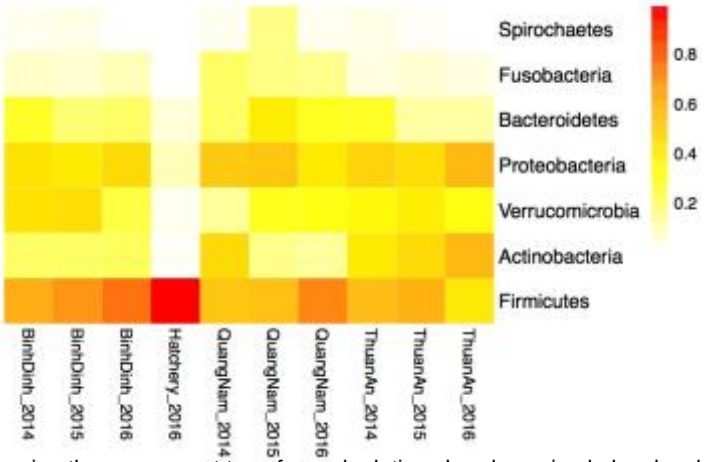

Figure 5.3. Heat map showing the square root transformed relative abundance in phylum level of the gut MC of the hatchery (Hatchery) and wild larvae from 3 locations (ThuanAn, QuangNam, BinhDinh) over 3 years. Weighted averages of the replicates are presented and no significant differences (ANOVA, n=10) were observed between replicates.

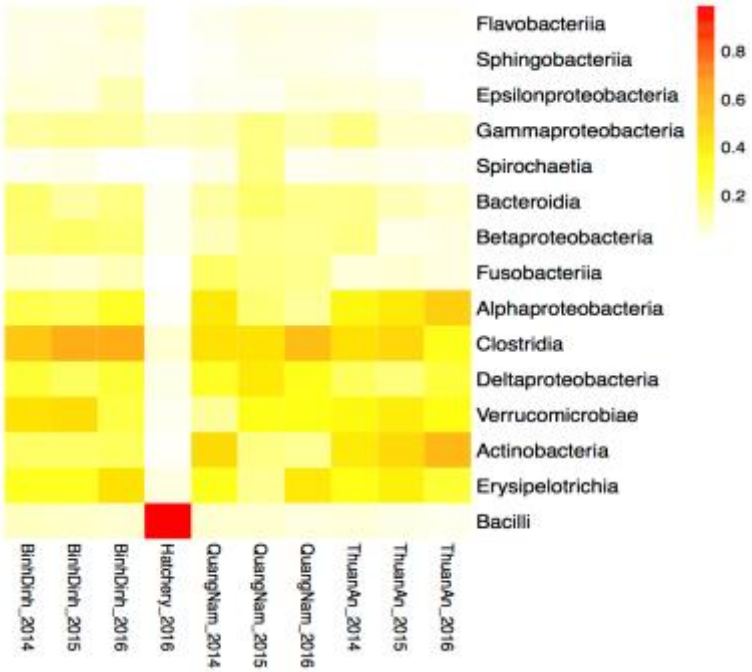

Figure 5.4. Heat map showing the square root transformed relative abundance in class level of the gut MC of the hatchery (Hatchery) and wild larvae from 3 locations (ThuanAn, QuangNam, BinhDinh) over 3 years. Weighted averages of the biological replicates are presented and no significant differences (ANOVA, n = 10) were observed between replicates.

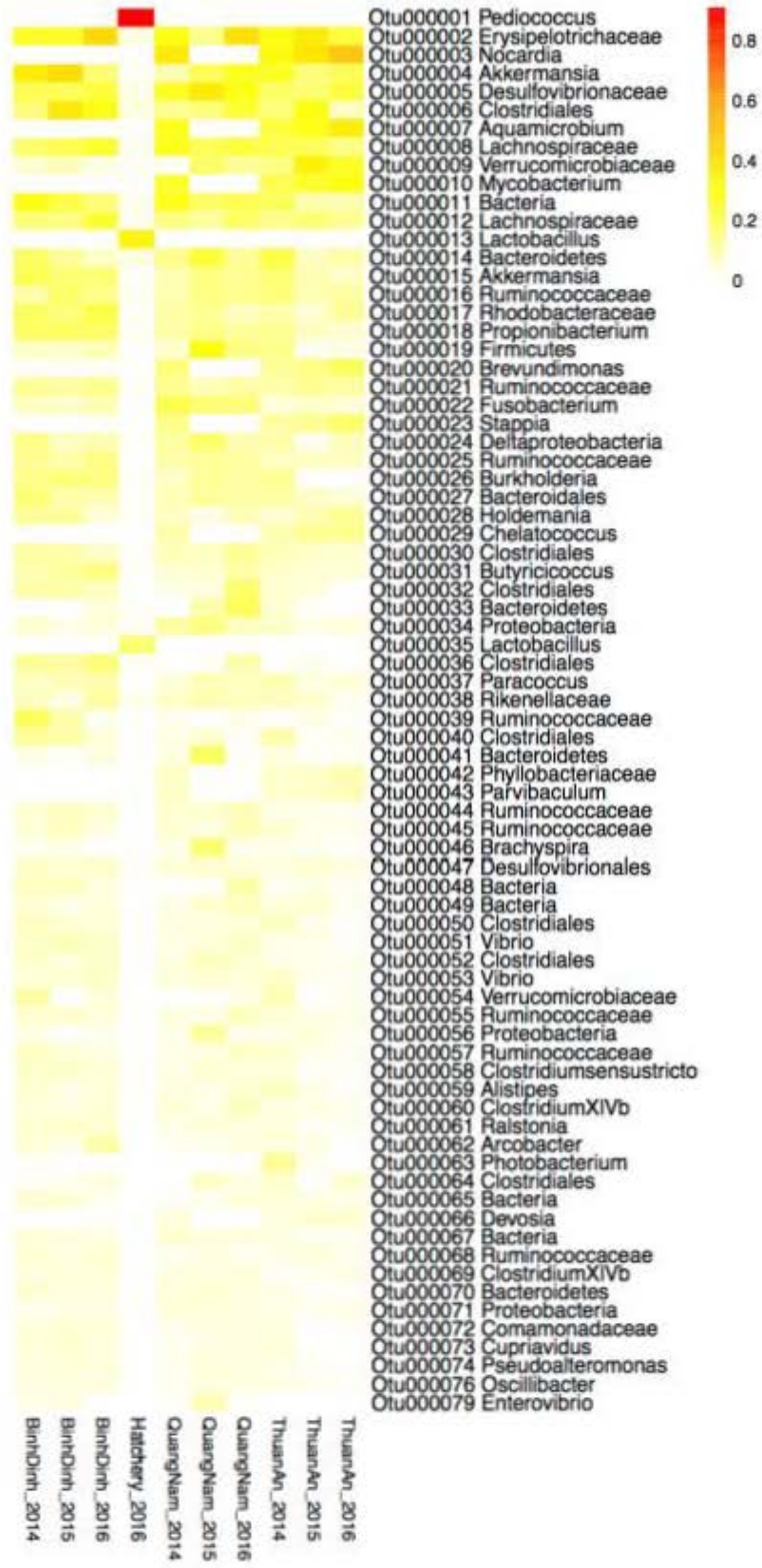

Figure 5.5. Heat map showing the square root transformed relative abundance in OTU level of the gut microbial of the hatchery-reared (Hatchery) and wild larvae from the three locations (ThuanAn, QuangNam, BinhDinh) over a three-year period. Weighted averages of the biological replicates are presented and no significant differences (ANOVA, n = 10) were observed between the biological replicates. The OTUs abundance of ≥ 0.1% were considered for analysis.

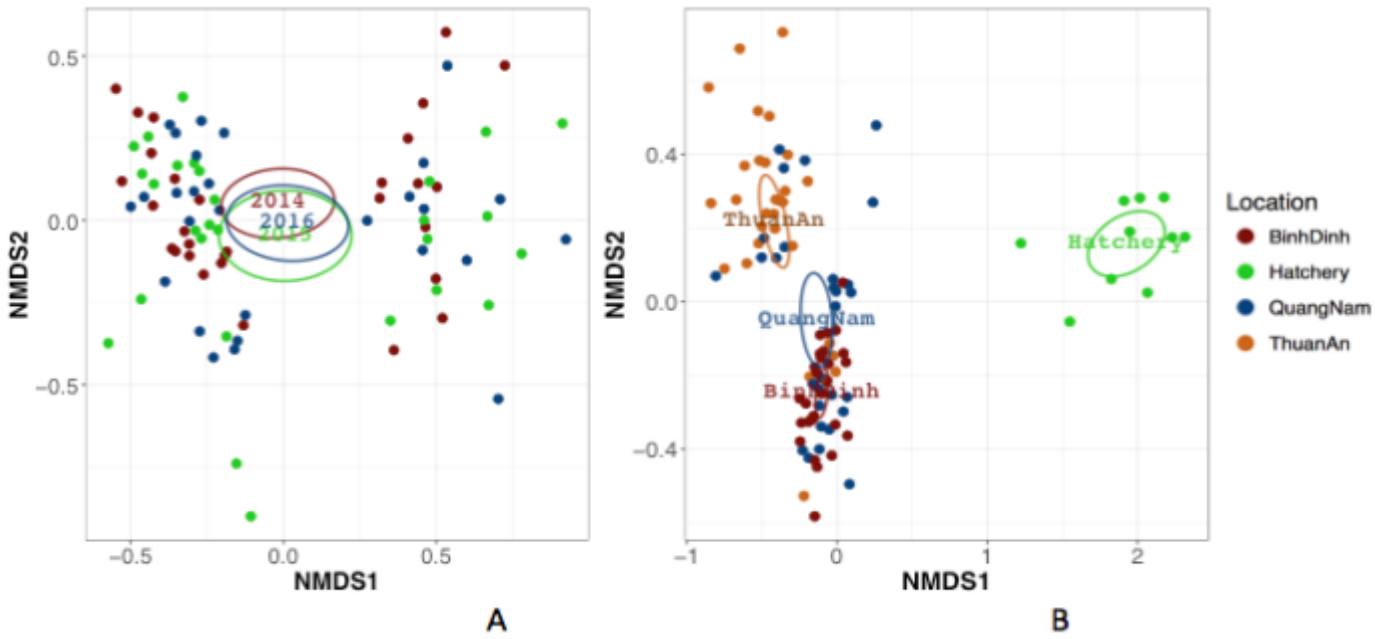

Figure 5.6. NMDS analysis of gut MC of hatchery and wild larvae from 3 different locations (ThuanAn, QuangNam, BinhDinh) over 3 years. A: distribution by years. B: distribution by location.

Table 5.4. Relative genetic distance between locations and its relation to temperature

No.	Location 1	Location 2	Relative genetic distance	
1	BinhDinh	QuangNam	0.42	
2	BinhDinh	ThuanAn	1.07	
3	QuangNam	ThuanAn	0.68	
	Factors		**p-value (PERMANOVA)**	**Spearman's rho**
1	Location and temperature		0.001	0.941

5.4. Discussion

The rabbit fish are herbivores, and they can be fed seaweed and aquatic plants for grow-out culturing. Although rabbit fish can synthesize PUFAs (Li *et al.*, 2010; Xie *et al.*, 2016), there is still lack of information about the importance of dietary PUFAs they require, especially in the larval phase. The understanding of the nutritional requirements of marine fish and rabbit fish in particular, especially their need for PUFAs is crucial for developing specific compound diets and/or live food enrichment protocols (Kitajima *et al.*, 1979; Watanabe *et al.*, 1982). The PUFA requirements are also important for designing brood stock diets that can optimize *e.g.* egg quality (Watanabe *et al.*, 1984a; Watanabe *et al.*, 1984b; Izquierdo *et al.*, 2001). This study showed that rabbit fish larvae contained a high proportion of ARA. The ARA content of wild larvae was stable between sampling locations over 3 years (between 1.96 and

4.19%), and no significant differences were detected in the ARA level between wild and hatchery samples. The ARA levels in rabbit fish of this study were similar to other studies on rabbit fish (Ogata *et al.*, 2004; Suloma and Ogata, 2011) and other rabbit fish, *e.g. S. virgatus*, *S. canaliculatus* (Ogata *et al.*, 2004; Suloma and Ogata, 2011; Monroig *et al.*, 2012), and *S. fuscescens* (Osako *et al.*, 2006; Jiarpinijnun *et al.*, 2017). The ARA content plays an important role in improving growth, survival and stress resistance of fish larvae (Bell *et al.*, 1997; Bessonart *et al.*, 1999; Tocher *et al.*, 2000; Koven *et al.*, 2001; Bell and Sargent, 2003). In fact, rabbit fish can synthesize ARA, since they have fatty acyl desaturases (Fads) in their body (Li *et al.*, 2010; Xie *et al.*, 2016). However, the main sources of body ARA content in wild larvae are still unclear. Macro algae (red and brown algae), phyto- and zooplankton are amongst the ARA-rich resources, which can be consumed by the wild and hatchery larvae (Johns *et al.*, 1979). The current study indicates that ARA content in hatchery rabbit fish larvae seems to be very similar to ARA levels found in wild larvae (over 3 years), indicating that hatchery larvae have no ARA deficiencies. It remains to be established in much more detail whether rabbit fish larvae are basically synthesizing sufficient ARA themselves using the current protocol or if at least part of the ARA content is acquired through the food. In marine fish species, the DHA/EPA ratio was reported to be variable between individuals, locations and sampling time points (Sargent and Whittle, 1981; Sargent *et al.*, 1999a; Sargent *et al.*, 1999b), *e.g.* in chum salmon (*O. keta*) (Sasaki *et al.*, 1989); red snapper (*L. argentimaculatus*), rabbit fish (*S. guttatus* and *S. canaliculatus*), coral trout (*Plectropomus leopardus*) and striped jack (*Caranx fulvoguttatus*) (Ogata *et al.*, 2004; Monroig *et al.*, 2012). The EPA content, DHA content and DHA/EPA ratio influence the reproductive performance of brood stocks, egg quality, embryo development, hatchability, larvae quality, and survival of marine fish larvae (Watanabe *et al.*, 1984a; Watanabe *et al.*, 1984b; Izquierdo *et al.*, 2001). In this study, the DHA/EPA ratios in wild larvae were very high. The ratios varied between locations and years. The DHA/EPA ratios in wild and hatchery larvae were first reported by Ogata *et al.* (2004). These values are very similar to those obtained in this study, either for the wild or hatchery larvae. In both studies, DHA/EPA ratios in hatchery larvae are located inside the range of DHA/EPA ratios found in wild larvae. Therefore, since the PUFAs of hatchery larvae are within the range of the wild larvae, the current larval rearing protocol is reliable in term of PUFA content and there seems to be no need to treat the hatchery larvae differently in the future (for instance there

seems to be no need to feed them HUFA enriched life food). In addition, difference between temperatures at the 3 locations is negatively correlated with the DHA content and the DHA/EPA ratio, but not with the EPA content. A negative correlation of DHA content with seawater temperature was reported between 15 and 30°C in the spotted pim (*Pimelodus maculatus*) (De Torrengo and Brenner, 1976), between 5 and 25°C in common carp (*C. carpio*) (Farkas *et al.*, 1980), between 22 and 29°C in European sea bass (*D. labrax*) (Skalli *et al.*, 2006), between 15 and 19°C in rainbow trout (*O. mykiss*) (Mellery *et al.*, 2016) and between 6 and 12°C in Atlantic salmon (*S. salar*) (Sissener *et al.*, 2017). In this study, while the EPA content of the rabbit fish remained stable, the fluctuation of DHA content resulted in the fluctuation in the DHA/EPA ratio in the samples from 3 locations over 3 years. During the early stage, temperature can impact the FA composition in 2 ways: (i) metabolism (efficiency and consumption rates) and (ii) homeoviscous adaptation (Sinensky, 1974). The increase of the DHA content as temperature decreases as caused by homeoviscous adaptation (additional DHA production for maintaining membrane fluidity for metabolic efficiency) mainly occurs in fish at low temperature, e.g. between 0 and 8°C in Pacific cod (*G. macrocephalus*) (Laurel *et al.*, 2012). Hence the current data rather point into the direction of temperature modulated activity of enzymes involved in PUFA metabolism. However, the temperature range across these 3 locations is narrow and hence more research could reveal a causal link.

Beside PUFA content, the fish gut MC has been reported to be involved in a wide variety of activities in fish physiology, such as digestive ability, uptake of nutrients, metabolism, signaling development and disease resistance (Nayak, 2010; Wong and Rawls, 2012). In earlier studies, the fish gut MC was investigated based on the measurement of phylogenetic relationships by Woese and Fox (1977). Since then, several molecular approaches for the gut MC studies were developed, such as 16S amplification followed by DGGE, TTGE, T-RFTL analysis (Romero and Navarrete, 2006; Hovda *et al.*, 2007; Navarrete *et al.*, 2009; Green *et al.*, 2013). In recent years, the rapid development in advanced molecular techniques, *e.g.* Illumina MiSeq, allowing researchers to conduct in-depth studies on the diversity, composition and ubiquity of the gut MC (van Kessel *et al.*, 2011; Geraylou *et al.*, 2013; Star *et al.*, 2013; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b; Zarkasi *et al.*, 2014; Gilbert *et al.*, 2015). This is the first study on rabbit fish gut MC by 16S amplification via Illumina

approach. In this study, we compared the gut MC of hatchery and wild larvae from 3 locations over 3 years to understand the potential relationship between locations and time with respect to the gut MC composition. The bacteria that were identified in the rabbit fish gut content were mainly belonging in the Proteobacteria, Bacteroidetes, Fusobacteria, Actinobacteria and Firmicutes phyla, which was similar to the studies on other fish gut MCs using 16S amplification on marine herbivores, *e.g.* whitecheek surgeonfish (*A. nigricans*), daisy parrotfish (*Chlorurus sordidus*), bulbnose unicornfish (*Naso tonganus*) and sixbar angelfish (*P. sexstriatus*); marine omnivores, *e.g.* black rockcod (*Notothenia coriiceps*) (Sullam *et al.*, 2012), blunt snout bream (*Megalobrama amblycephala*) (Li *et al.*, 2014); marine carnivores, *e.g.* blackfin icefish (*Chaenocephalus aceatus*), long-snout seahorse (*H. guttulatus*), two-spot red snapper (*L. bohar*), sole (*S. senegalensis*) and grass puffer (*Takifugu niphobles*); estuarine carnivores, *e.g.* grouper (*E. coioides*) and longjaw mudsucker (*Gillichthys mirabilis*) (Sullam *et al.*, 2012); freshwater herbivores, *e.g.* grass carp (*C. idellus*) (Li *et al.*, 2014; Liu *et al.*, 2016); freshwater omnivores, *e.g.* zebra fish (*D. rerio*), guppy (*Poecilia reticulata*) (Sullam *et al.*, 2012), common carp (*C. carpio*); silver carp (*H. molitrix*), bighead carp (*H. nobilis*), mandarin fish (*Siniperca chuatsi*) (Li *et al.*, 2014; Liu *et al.*, 2016); freshwater carnivores *e.g.* rainbow trout (*O. mykiss*), yellowhead catfish (*Pelteobagrus fulvidraco*), Atlantic salmon (*S. salar*), brown trout (*S. trutta*) (Sullam *et al.*, 2012; Li *et al.*, 2014; Dehler *et al.*, 2017). Moreover, OTUs from *Vibrio sp.* were up to 1.1% in the gut MC composition of the wild larvae, which is similar to the *Vibrio sp.* prevalence is the gut of other fishes, *e.g.* cod larvae (approx. 1%) (Bakke *et al.,* 2015). No OTUs from *Vibrio sp.* were found in the gut MC of hatchery larvae. It might be due to the dominance of LAB and *B. subtilis* in the gut MC of hatchery larvae. The dominance of LAB and *B. subtilis* in the gut MC could limit the adhesion process of bacteria pathogens to the epithelium (Olsson *et al.*, 1992; Spencer and Chesson, 1994; Jin *et al.*, 1996; Ouwehand and Conway, 1996; Bomba *et al.*, 1997; Jöborn *et al.*, 1997; Velraeds *et al.*, 1997; Hansen and Olafsen, 1999; Merrifield *et al.*, 2010; Pérez *et al.*, 2010; Merrifield and Ringø, 2014; Hoseinifar *et al.*, 2016). Also, the presence of LAB and *B. subtilis* in the gastrointestinal tract and eventually gut mucosa could provide antagonistic activity, by producing specific compounds such as lactic and other organic acids. These compounds inhibited the proliferation of other proteolytic bacteria, thus protecting the fish from bacterial pathogens (Shahani *et al.*, 1977; Hurst, 1981; Stoffels *et al.*, 1992; Merrifield *et al.*,

2014; Hoseinifar *et al.*, 2016). Some LAB and *B. subtilis* produce bacteriocins, bactericidal or bacteriostatic peptides, which are antibacterial substances (Lewus *et al.*, 1991). *B. subtilis* also produces small molecular siderophores (<5 kDa) which have a wide spectrum of activity against bacteria pathogens in the fish intestine (Sugita *et al.*, 1996; Sugita *et al.*, 1998). It remains to be established to what degree the used probiotics interfere with the gut MC through these mechanisms.

The differences in the gut MC between 3 locations might relate to the differences in temperatures, current direction and food quality. Firstly, there is a positive correlation of temperature differences at the 3 locations over 3 years with the genetic differences between gut MC. Significant impact of environment temperature on the gut MC has been reported not only in other fish species (*e.g.* silver carp (*H. molitrix*) (Ye *et al.*, 2014), but also in the other ectotherm animals (*e.g.* tadpoles (Kohl and Yahn, 2016)). Secondly, the flow direction of water currents in Central Vietnam can explain the correlation of genetic distances of the MC and locations. In June, the currents usually flow from BinhDinh to ThuanAn and the currents are partially blocked by the Hai Van pass (peninsular mountains), which are located between ThuanAn and QuangNam (MARD, 2016a). The change of the current direction might create differences in the water bodies between 3 locations, hence affecting the water MC and resulting in the separation of the gut MC in ThuanAn from other locations in the south (QuangNam and BinhDinh). Lastly, it might be that difference in the food quality at the 3 locations is shaping the MCs and difference in cellulose content of the food is potentially important in shaping the MC. In summary, location seems to shape gut MC. Several abiotic factors such as water temperature, water current, quality of the local food could be the driving factors, alone or in combination. A more extensive monitoring program in term of gut MC characterization and abiotic factors in combination with experimental approaches could reveal which abiotic factors is the main driver of the gut MC of rabbit fish.

The gut MC composition of wild and hatchery larvae in this study was completely different. This can be explained by the special conditions such as feeding and probiotic additions in the hatchery. During the rearing, the hatchery larvae were fed probiotic-enriched rotifers from first feeding until sampling. The probiotics added daily to rotifers culture included *L. plantarum, L. fermentum, L. brevis, P. acidilactici, P. pentosaceus* and *B. subtilis* (Bose, 2011; Le et al., 2017) at 10^6 CFU.mL^{-1} for each strain.

Apparently wild larvae did not take up these species through their natural food. Secondly, the biotic factor, which is the trophic level of rabbit fish in this study, might impact the gut MC. At the time the rabbit fish larvae migrate to the river mouth (14-18 mm in length, which is equivalent to the 25-day old larvae in the hatchery), they already become herbivores and are predominantly grazing on seaweed and aquatic plants (Pillai, 1962; Rosario, 1975; Alcala and Alcazar, 1979; Alcala, 1979; Tseng and Chan, 1982; Anon, 1983; Gundermann *et al.*, 1983; Urmaza Sr, 1983; Kishimoto, 1984; Lichatowich *et al.*, 1984; Juario *et al.*, 1985; Hara *et al.*, 1986c). However, low trophic level herbivores, which consume aquatic plants as food, usually lack cellulase. As a result, they need cellulase-producing bacteria to convert part of the food into short-chain fatty acids. Hence, the gut MC is important for the herbivorous fish to break down indigestible food components into digestible components and nutrients (Saha *et al.*, 2006; Ray *et al.*, 2012). Indeed, the gut MC of herbivores also contained a high relative abundance of potentially cellulose-degrading bacteria, belonging to the orders Clostridiales and Fusobacteriales (Liu *et al.*, 2016). In our study, these orders were dominant in the wild larvae, but it was not the case in the hatchery. The trophic level of hatchery larvae was kept differently from the nature. By feeding probiotic-enriched rotifers to the larvae, they were forced to feed as carnivores. In summary, rabbit fish larvae can harbor totally different gut MC and be apparently healthy (wild versus hatchery). Hence, gut MC must be shaped by the prevailing environmental conditions, which in the wild might be food, temperature and local water MC. In the hatchery, it is shaped through continuous supply of probiotics via live food (rotifers).

This is the first study to compare the fish gut MC of wild and hatchery larvae of a marine herbivorous species in general, and of rabbit fish in particular. This study contains baseline scientific data. It might be useful for the establishment of protocols for larval rearing and growth out of rabbit fish. For instance as the hatchery larvae, fed live feed, will need to switch to natural food upon stocking in ponds, it can become of interest to verify if gut MC management is of importance in the process (switch from hatchery to pond).

CHAPTER 6

General discussion and conclusions

6.1. Context of this study

Rabbit fish (*S. guttatus*) larviculture has been targeted by aquaculturists since the 1980s. However, rabbit fish larvae are difficult to rear in the first feeding period (from hatching up to 6 days) because of their small mouth size at opening and first feeding, and its small yolk-sac volume (Bagarinao, 1986; Hara *et al.*, 1986a; Duray, 1998b), which results in low survival from hatching to day 24 (0 – 1.1%) (Gorospe *et al.*, 2011).

In the early development of fish larvae, the transition from endogenous to exogenous feeding is a critical period. In the artificial condition, mass mortality was observed at this stage. The survival of fish larvae is variable depending on fish species, abiotic factors, age of brood stocks, egg quality, MC, spawning and rearing conditions (Lillelund, 1965; Blaxter, 1969; May, 1974; Kjørsvik *et al.*, 1990; Tamaru *et al.*, 1994; Brinkmeyer and Holt, 1998; Olsen *et al.*, 1999; Baskerville-Bridges and Kling, 2000; Hamlin and Kling, 2001; Olafsen, 2001; Puvanendran and Brown, 2002; Cahu *et al.*, 2003; Yúfera *et al.*, 2005; Palazzi *et al.*, 2006; Yúfera and Darias, 2007; Bobe and Labbé, 2010). The survival of fish larvae in the first couple of weeks after mouth opening and first feeding ranged from the best survivals recorded of 91% in sea bream (*S. aurata*) (Yúfera et al., 2005; Yúfera and Darias, 2007*) to* 85% in Senegal sole (*S. senegalensis*) *(*Yúfera et al., 2005; Yúfera and Darias, 2007)*,* 82% in grey mullet (*M. cephalus*) (Tamaru et al., 1994)*,* 75% in European seabass (*D. labrax*) (Cahu et al., 2003), 60% in common sole (S. solea) (Palazzi *et al.*, 2006); 46% in red drum (*S. ocellatus*) *(Brinkmeyer and Holt, 1998),* 41% in Atlantic cod (*G. morhua*) (Baskerville-Bridges and Kling, 2000; Puvanendran and Brown, 2002); 38% in haddock (*M. aeglefinnus*) (Hamlin and Kling, 2001) *and* 25% in Atlantic halibut (*H. hippoglossus*) *(Olsen* et al.*, 1999).* The main reasons for the mass mortalities of fish larvae at first feeding stage were identified as (i) lack or insufficient food at first-feeding in nature; (ii) low quality of first-feeding food; (iii) changes in environmental conditions and (iv) microbial interference (Hunter, 1981). Especially food quality was an important factor affecting the survival of larvae (Kaji *et al.*, 1996; Fernandez-Diaz and Yúfera, 1997; Cahu *et al.*, 1998; Hamlin and Kling, 2001; Robin and Vincent, 2003; Papandroulakis *et al.*, 2005; Yúfera *et al.*, 2005). It is important to understand the early development of fish larvae in terms of nutritional requirements. The duration of the period of the larvae transition from endotrophic to exotrophic life is largely dependent on the water temperature and the yolk-sac volume of the larvae (Howell, 1980; Bagarinao, 1986; Arul, 1991; Polo *et al.*, 1991; Buckley *et al.*, 2000; Parra and Yúfera, 2001; Hardy and

Litvak, 2004). In order to achieve high survival, it is necessary to find a food which is suitable in size and composition, thus matching the mouth size of larvae and the development of the digestive system (Yúfera and Darias, 2007). More importantly, the energy obtained from the food should be higher than the energy spent by the larvae for catching the food. Moreover, feeding behavior and feeding incidence were also considered as important factors for the performance of fish larvae at first feeding (Bagarinao, 1986; Yúfera and Darias, 2007). The feeding behavior of larvae is depended on the food density, the food distribution, and the larvae aggressiveness towards the food. The term of Point of No Return (PNR) was used to indicate the inability of 50% of larvae to catch food actively in the first feeding stage. At this point, the larvae were still alive but too weak to catch food even when food became available. The PNR can also be called “irreversible starvation” (Yin and Blaxter, 1987). Each marine fish species has its own PNR, which ranges from 3 days to 20 days. A delay in the introduction of food in this period might result in high deformity, low feeding percentage, digestive problems and low survival of the fish larvae (Yin and Blaxter, 1987; Miller *et al.*, 1988; Arul, 1991; Yúfera *et al.*, 1993; Gwak and Tanaka, 2001; Dou *et al.*, 2002; Gisbert *et al.*, 2004). In conclusion, it is crucial to find a proper-sized live food for each type of fish larvae to avoid the PNR.

Within this Ph.D. study, we focused on the critical food size and probiotics affecting the larviculture of rabbit fish. In the first experiment, we aimed at verifying the importance of the proliferating bacterial community on the growth of very small rotifers *P. similis*. Subsequently, a new protocol of the large-scale culture of *P. similis* was established (chapter 3). Profiting from the technology of large-scale culture of *P. similis*, in chapter 4, we studied the effects of different live food size and different type of live food combined or not with probiotics addition on rabbit fish larval rearing from hatching to day 25. The experiments included a co-feeding approach including live food: *P. similis, B. rotundiformis*, *B. plicatilis*, *Artemia* and compound diets as it was anticipated that co-feeding would yield good results. The LC-PUFA composition and intestinal MC of wild rabbit fish larvae from 3 different locations in Central Vietnam over 3 years, and the hatchery larvae were compared in order to understand the nutritional requirements and gut MC of the rabbit fish larvae (chapter 5). It is anticipated that this knowledge will allow to further optimizing the larviculture.

6.2. The addition of proliferating bacterial community to optimize the culture of the very small rotifers (*P. similis*)

In previous studies, microalgae were reported to be the most appropriate food for the culture of *P. similis* (Wullur *et al.,* 2009, 2011). After that, Wullur *et al.* (2011) described a large-scale culture of *P. similis* in 50L tanks, to provide enough rotifers for rearing the larvae, using very small microalgae species such as *N. oculata* and *C. vulgaris.* However, *P. similis* showed a slower growth rate during the first 4 days than in the later stage (Wullur *et al.* 2009). It was hypothesized that, the bacterial community might have some possible influence on the growth performance of the *P. similis*, perhaps not directly, but nevertheless very important. In the present study, the effect of limiting the bacterial re-growth, through the addition of an AB mixture, and the effect of the addition of a small amount of live or dead bacteria (a mixture of LAB and *B. subtilis*) on the population growth, growth rate and MC of *P. similis* culture was investigated for a period of 10 days. For comparison purpose, the bigger rotifer *B. rotundiformis* culture was included as a control. The results showed that in the presence of a proliferating bacterial community, both rotifers species showed a better population growth rate than those without the presence of a proliferating bacterial community. In addition, the growth performance of the smaller rotifer *P. similis* proved to be more dependent on a proliferating bacterial community than the bigger rotifer *B. rotundiformis*. This proliferating bacterial community not only increased the production of the rotifers but also had a regulating effect on the MC composition of rotifer culture water. The importance of bacteria in rotifer cultures was first reported by Starkweather *et al.* (1979), when the rotifer *B. calyciflorus* were cultured on the bacterium *A. aerogenes* under laboratory conditions. Although the feeding rates on bacteria were lower compared to bigger particles, it was found that this rotifer species can be cultured for more than 40 generations solely on *A. aerogenes* without any noticeable negative effects on reproductive rate or lengthening of the cohort generation time (Starkweather *et al.*, 1979). Later, Arndt (1993) reported that bacteria could be a substantial part of the food for certain rotifer species, namely belonging to the Brachionidae. Depending on the mixture of available food particles, 10 - 40% of the rotifer diet could consist of bacteria. However, as the grazing rate was low compared to the bacteria growth rates, the effect on the bacteria population in the culture tank will be low (Arndt, 1993). In addition, Agasild and Nõges (2005) stated that in the conditions with a limited amount of microalgae, rotifers could switch to the uptake of bacteria-sized particles for food. Some bacteria, such as *Pseudomonas* species, can synthesize vitamin B_{12} and subsequently be beneficial to rotifers (Yu *et al.*, 1988). Rotifer culture in bacteria-

free conditions showed lower population growth and growth rate than those in the culture with the presence of bacteria. It meant that bacteria were one of the important factors, which contributed to the reproduction of rotifers (Douillet, 1998; Rombaut *et al.*, 1999; Tinh *et al.*, 2006). Among many bacteria strains used to manipulate the MC in rotifer culture, LAB and *B. subtilis* appeared to be the most popular. Live LAB and *B. subtilis* were reported to enhance the production of the rotifer *B. plicatilis* (Gatesoupe, 1991b; Harzevili *et al.*, 1998a; Hirata *et al.*, 1998; Douillet, 2000). The addition of *L. casei, P. acidilactici* and *L. lactis* increased the growth rate of *B. plicatilis* by 8-13 times compared to those obtained in the controls. In this study, 5 strains of LAB and 1 strain of *B. subtilis* were used to investigate the effects on the maximum population density, growth rate and MC of *P. similis* and *B. rotundiformis*. The results showed that the proliferating bacterial community was a very important factor in the growth performance of both *P. similis* and *B. rotundiformis* cultures, pointing in the direction of the importance of the microbial loop. In comparison to the control without AB, the results seem to indicate that the proliferation of probionts in the cultures is very beneficial which can be due to the combination of additional available bacterial biomass (nutritional effects) and probiotic effects. However, the data did not allow us to quantitatively distinguish the effects from both factors. To quantify the contribution of bacteria biomass to the growth of *P. similis*, different types of experiments should be performed. The standing microbial and algal community could be quantified by *e.g.* flow cytometry and supplemented (spiked) with labelled (^{3}H or ^{13}C) probiotics in a short-term experiment (*e.g.* 1h). Subsequently, label incorporated into *P. similis* biomass could be determined. The data however allowed us to conclude that the rotifer *P. similis* is more dependent on the proliferating bacterial community than the rotifer *B. rotundiformis* in both experimental and large-scale culture conditions.

Finally, a large-scale culture protocol (200 L) of *P. similis* using proliferating bacteria was established. It is recommended to add live bacteria, *e.g.* LAB and *B. subtilis* daily, at 10^{6} $CFU.mL^{-1}.species^{-1}.day^{-1}$ to the rotifer cultures to enhance the rotifer production, improve the MC composition and reduce the culture period from 11 to 6 – 8 days.

6.3. Larviculture of rabbit fish (*S. guttatus*)

Rabbit fish is an important cultured species in tropical countries. In chapter 4, new larval rearing protocols were designed, based on using *P. similis* as first food for the rabbit fish larvae. The study on the larval rearing of rabbit fish (from hatching to 25 dph) was divided into 2 phases: (i) the first feeding phase was accounted from hatching to day 10 and (ii) the second phase was delineated from day 10 to 25 (Duray, 1998b). In the first experiment, we aimed to answer the question on whether the rabbit fish could survive up to 240 hph using *P. similis* or *B. rotundiformis* as starter food. In this study, the larvae fed smaller rotifer *P. similis* had significantly higher survival at 240 hph than those fed the bigger rotifer *B. rotundiformis* as starter food. Indeed, the effect of live food size on fish larvae with small mouth/special oesophagus at first feeding was reported for many marine fish species, such as the seven-band grouper, rusty angelfish, humphead wrasse*;* and Japanese eel (Shirota, 1970; Fernández-Díaz *et al.*, 1994; Busch, 1996; Munk, 1997; Planas and Cunha, 1999; Østergaard *et al.*, 2005; Yúfera and Darias, 2007; Wullur *et al.*, 2009; 2011; Hirai *et al.*, 2012; Hirai *et al.*, 2013; Wullur *et al.*, 2013; Hagiwara *et al.*, 2014). This experiment showed that also for rabbit fish the very small rotifer *P. similis* is a better starter food than the bigger rotifer *B. rotundiformis*.

In the previous studies on rabbit fish larval rearing in the Philippines, most of the rotifers, which belonged to the group *B. plicatilis* complex, were still too big on 6 dph (Hara *et al.*, 1986c; Kohno *et al.*, 1988; Ayson, 1989). Several studies explained that during the first few days of larval development, a rapid increase of mouth size (jaws) certainly helped the larvae to catch a bigger size of food and to increase ingestion rate, increasing the survival (Shirota, 1970; Polo *et al.*, 1992; Fernández-Díaz *et al.*, 1994; Doi *et al.*, 1997; Olsen *et al.*, 2000). In order to increase the survival, the second experiment was conducted testing several co-feeding schemes of *P. similis* and *B. rotundiformis*.

In general, the combination of food of two different sizes always resulted in increased survival, compared to the mono-diet scheme. Previous studies showed that the food size had a great impact on the growth and survival of the fish larvae in general, and the ratio of the food over mouth size should be ranging from 0.2 to 0.7 depending on species and the stage of larval development. The mouth size of rabbit fish and its function at early stage of development are indicated in Table 6.1.

Table 6.1. Rabbit fish mouth size and its function at early stage.

Age (hph)	Mouth size (µm)	Food size (µm)	Function	References
30.5 – 32	80 – 110	16 – 77	Mouth opening, learning how to eat	Ordonio-Aguilar (1995); Duray and Kohno (1990)
36	125	25 – 88	Start to eat *P. similis* (40 – 80µm)	This study
55.5 – 60	200	40 – 140	Start to eat *B. rotundiformis* (90 – 150µm)	Duray and Kohno (1990); This study
82.5	219	44 – 153	Start to eat *B. plicatilis* (142 – 203µm)	Kohno *et al.* (1988); Hara *et al.* (1986a)

Moreover, a ratio close to the upper limit of the range was ideal for food uptake by the larvae (Shirota, 1970; Fernández-Díaz *et al.*, 1994; Busch, 1996; Munk, 1997; Planas and Cunha, 1999; Østergaard *et al.*, 2005; Yúfera and Darias, 2007). It is easier for the fish larvae to have a net energy gain by catching bigger slow moving prey, which is still within the limits of their mouth size (Hunt von Herbing and Gallager, 2000). The highest larvae mortality occurs in the few days or weeks after hatching as a larva shifts from endogenous (reliant on yolk) to exogenous feeding (reliant on external prey) (Hewitt and George, 1987; Houde, 1989). During this time, relatively slow-swimming larvae may be unable to capture fast-moving preys; food limitation may result in starvation after the yolk has been absorbed. Lack of appropriate prey may be the most important factor influencing larval fish survival (Hunt von Herbing and Gallager, 2000). The attack rate and successful ingestion by the larvae are positively correlated with the larval length and age. When food was totally absent, the initial increase in larval length was absolutely dependent on the preserved energy of the yolk. In case the food density is low or the food size is small, the swimming time is increased in order to maintain the ingestion threshold (Munk and Kiorboe, 1985; Puvanendran and Brown, 2002). Therefore, size and energy content of the food were an important factor affecting the larval survival. In fact, the DW of *B. rotundiformis* is 6-fold higher than *P. similis*. It was assumed that larvae feeding on the bigger *B. rotundiformis* obtained more energy-per-catch than those feeding on the smaller *P. similis* (Wullur *et al.*, 2011). From 5 dph, the mouth and body size (Bagarinao, 1986; Hara *et al.*, 1986b) of larvae increased, *B. rotundiformis* should be added and *P. similis* should be gradually replaced to obtain a positive energy balance for the larvae.

The co-feeding regime in experiment 2 (chapter 4) leads to good survival of larvae at 240 hph (13.7%) compared to the current methods used in aquaculture. In this experiment, both rotifer species were cultured without any bacteria enrichment.

However, when probiotic-enriched rotifers were used, the larval survival increased from 13 to 16% at 240 hph (experiment 3, chapter 4), and this survival was higher than in the previous report from a culture in the Philippines in 2011 (<1% survival) (Gorospe *et al.*, 2011). The effect of LAB and *Bacillus sp.* via feeding to marine fish larviculture is summarized in Table 6.2. It seems that the probiotic-enriched rotifers were the key factor for the significantly higher survival of the fish larvae. The probiotics delivered to the fish larvae through their feeding activity on *e.g.* probiotic-enriched rotifers improves the larval performance. In term of probiotic traceability, LAB and *Bacillus sp.* used for bacteria bioencapsulation in live food (via rotifers and *Artemia,* continuous feeding) could be traced in the gut MC of seabass (*D. labrax*) (Picchietti *et al.*, 2009) and seabream (*S. aurata*) (Suzer *et al.*, 2008; Avella *et al.*, 2010; Arığ *et al.*, 2013) This was not the case for the grouper (*E. coioides)* (via copepods, continuous feeding) (Sun *et al.*, 2013). There was no information in the probiotic traceability in the rabbit fish *(S. rivulatus)* (via pellets) (El-Dakar *et al.*, 2007), turbot (*S. maximus*) (via rotifers and Artemia) (Gatesoupe, 1989; Garcia-de-la-Banda I. *et al.*, 1992), Dentex (*D. dentex*) (via pellets) (Hidalgo *et al.*, 2006).

Table 6.2. Application of LAB and *Bacillus sp.* via live food to marine fish larviculture

Probiotics	Target species	Food	Traceability	References
B. subtilis	Rabbit fish *(S. rivulatus)*	pellets	N/A	El-Dakar *et al.* (2007)
Bacillus sp.; *B. toyoi*; *L. plantarum, L. helveticus L. bulgaricus*	Turbot (*S. maximus*)	rotifers, *Artemia*	N/A	Gatesoupe (1989); Garcia-de-la-Banda I. *et al.* (1992)
L. delbrueckii, B. subtilis	Seabass (*D. labrax*)	rotifers, *Artemia*	Yes	Carnevali *et al.* (2006); Picchietti *et al.* (2009); Md *et al.* (2015)
Lactobacillus sp., Bacillus sp.	Sea bream (*S. aurata*)	rotifers	Yes	Suzer *et al.* (2008); Avella *et al.* (2010); Arığ *et al.* (2013)
B. pumilus, B. clausii	Grouper (*E. coioides)*	copepod	No	Sun *et al.* (2013)
B. toyoi, B. cereus	Dentex (*D. dentex*)	pellets	N/A	Hidalgo *et al.* (2006)

There were several studies on the presence of probiotics on the fish gut MC using new generation sequencing method (Illumina HiSeq high-throughput sequencing of 16S rRNA gene). In fact, LAB can be found in the gut MC of farmed species fed compound diet, e.g. rainbow trout (*O. mykiss*) (up to 4% of OTU abundance) (Ingerslev *et al.*, 2014b) and Atlantic salmon (*S. salar*) (Dehler *et al.*, 2017), however, they cannot be detected in wild samples, e.g. common carp (*C. carpio*), silver carp (*H. molitrix*),

bighead carp (*H. nobilis*), mandarin fish (*Siniperca chuatsi*), grass carp (*C. idellus*) (Liu *et al.*, 2016). In fact, the compound diet might contain probiotics and hence the probiotics can be traced from the fish gut content, however, this is not the case in the wild samples.

The probiotic examination of gut microbiota during the larval stage (from 1 to 49 dph) using Illumina HiSeq high-throughput sequencing of 16S rRNA gene was first reported on rainbow trout (*O. mykiss*) (Ingerslev *et al.*, 2014b), and was compared to the results from our study (Table 6.3). It seems that the feeding of powdered *P. acidilactici* via compound diet resulted in low abundance of *P. acidilactici* OTUs in the gut MC of the rainbow trout; even when they are fed probiotics continuously. In our study, the putative LAB and *Bacillus sp.* were used for long-term enrichment via rotifers and were fed continuously to the larvae. They could be traced and were eventually predominant in the fish gut MC. In conclusion, it seems that the combination of continuous putative probiotic enrichment via live food to the larvae improve the traceability of probiotics (LAB and *Bacillus sp.*) from the fish gut MC.

Table 6.3. Examination of probiotics in the gut MC of fish larvae using Illumina HiSeq high-throughput sequencing of 16S rRNA gene

Components	Rabbit fish	Rainbow trout
Probiotics	5 strains of LAB, 1 strain of *B. subtilis*	*P. acidilactici*
Type	putative (growth on MRS broth)	powder (Bactocell®)
Probiotic OTUs abundance in gut MC	up to 99%	up to 7%
Food use	rotifers, Artemia	compound diet
Continuous supply of probiotics	yes	yes
Sampling day	25	26
Reference	This study	Ingerslev *et al.* (2014b)

Lastly, the effect of the feeding protocol for the larval rearing from day 10 to day 25 on the larval survival was also studied (experiment 4, chapter 4). The co-feeding *Artemia* and rotifers gave the best larval survival (94%). The second best larval survival was observed in the larvae co-fed *Artemia*, rotifers and pellets, 84%). When *Artemia* was not used in the feeding scheme, the survival was lower than treatments with *Artemia* addition. It indicated that *Artemia* has a crucial role in rabbit fish larval rearing after day 10, as it became the most important food of the larvae (Hara *et al.*, 1986a). The

advantages of *Artemia* in the second stage of larval rearing of rabbit fish were earlier reported by Duray (1998b) and Ayson *et al.* (2014). The lowest survivals were obtained in the treatment without rotifers addition, 44%. These results showed that besides *Artemia*, rotifers were also important for the larval rearing at this stage. Within a fish larval batch, the smaller larvae would need rotifers as an alternative food until they can reach the size, that can catch *Artemia*. Duray (1998b) and Ayson *et al.* (2014) suggested that rotifers should be included in the feeding scheme up to day 20 for siganids larval rearing. In terms of economic aspects, addition of compound diet could be considered in the larval rearing because the price per kg of *Artemia* is 20-fold higher than for compound diet. Therefore, operational cost could be reduced by compound diets inclusion into the feeding scheme. From this study, it is suggested that pellets (compound diets) can be co-fed with rotifers and Artemia for cost-effectiveness.

In summary, we recommend a feeding scheme for the larval rearing of rabbit fish from day 0 to 25 (Figure 6.1). Basically, from hatching to 240 hph, the very small rotifer *P. similis* must be used as primary food. The bigger rotifer *B. rotundiformis* should be introduced from day 6. At this point the biomass of *P. similis* should be reduced. From day 10 to day 25, rotifers are still important in the diets of the larvae. At this stage, as the larvae grow, bigger rotifer (*e.g.* L-type *B. plicatilis)* should be provided. All 3 rotifers species (*P. similis, B. rotundiformis* and *B. plicatilis*) should be enriched with probiotics prior to feeding to the larvae. *Artemia* nauplii (Vinh Chau, Vietnam) become effective when introduced from day 11. More importantly, there is no obligation to enrich the live food (rotifers and *Artemia*) with PUFAs prior to feeding to the larvae, as the rabbit fish themselves can synthesize PUFAs and stay healthy. Pellets (compound diets) can be co-fed with rotifers and *Artemia* for the cost-effectiveness. There are basic differences in the feeding scheme recommended for the larval rearing of the rabbit fish (small mouth size at opening, 80 µm) (this study), compared to those from the Asian seabass (big mouth size at opening – 220 µm) (Duray and Juario, 1988). With the much bigger mouth size at opening, the Asian seabass can immediately consume the L-type *B. plicatilis* as starter food. Also, *Chlorella sp.* was used following the green water technique. Hence, it is not necessary to use *P. similis* as starter food for the Asian seabass. The use of *Artemia* and compound diet is apparently similar in both fish species. The current protocol was applied on a larger production scale in 3 different

trials (in 3 different months) in 4,000 L culture tanks (containing 2,000 L of seawater at stocking, totally 40,000 larvae.tank^{-1}). The production was stable at 5,000 – 6,000 larvae.tank^{-1} at day 25 and between 15,000 – 20,000 larvae were produced in total for grow-out culture (to the farmers and others).

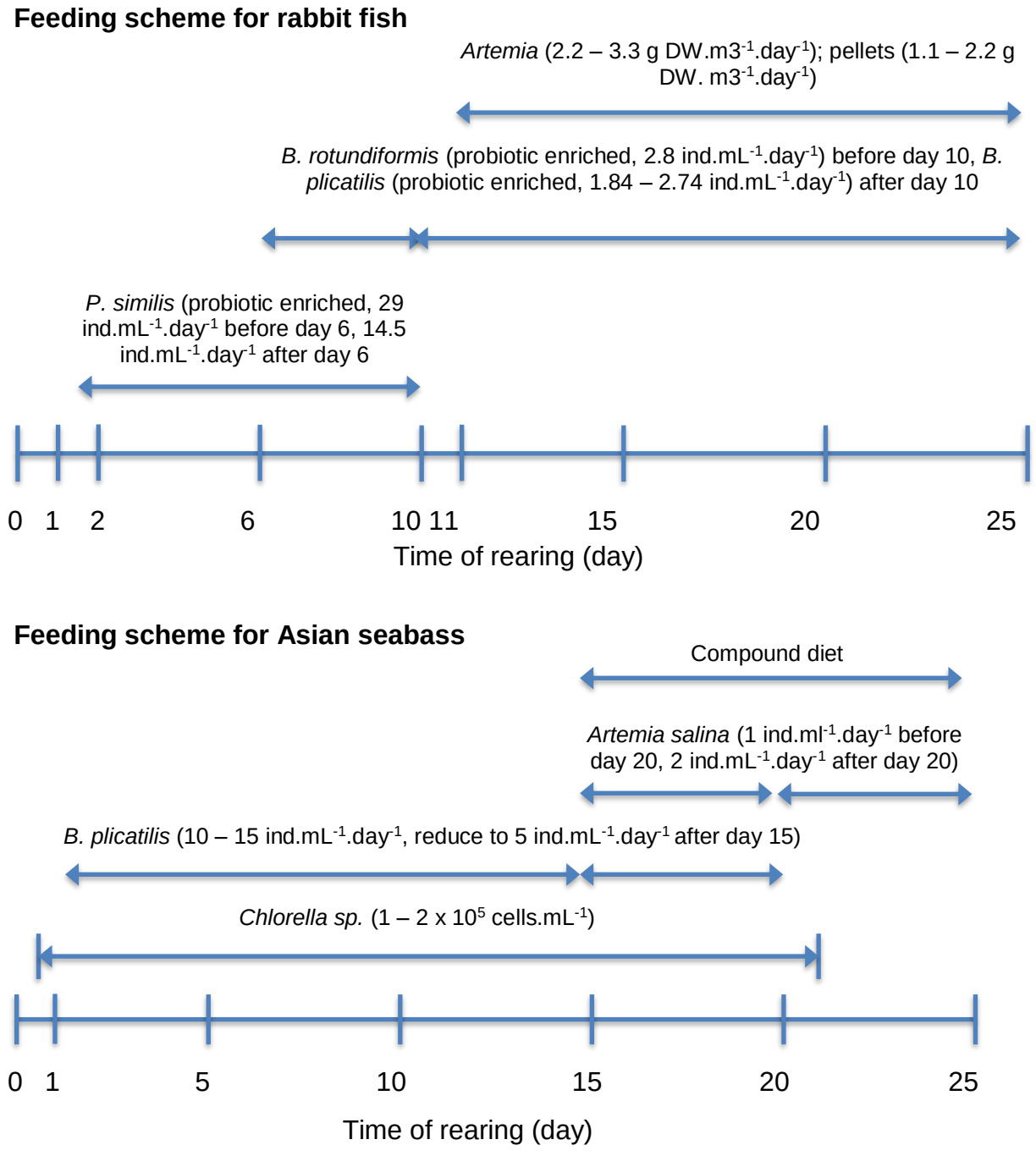

Figure 6.1. Feeding schemes for the larval rearing of fish with small mouth size – rabbit fish (*S. guttatus*) (this study) and big mouth size – Asian seabass (*L. calcarifer*) (Duray and Juario, 1988)

6.4. PUFA and gut MC composition of wild rabbit fish larvae from 3 sampling locations over 3 years and from the hatchery

In order to understand the larval nutritional requirements, the PUFA composition of the wild and hatchery larvae was determined in this study. The wild larvae were collected in 3 different locations over 3 years in the Central Vietnam where the migrating rabbit fish have been found abundantly. Results of this study showed that both wild and hatchery larvae contained a high proportion of ARA and DHA. The DHA/EPA ratios were also very high (3:1 – 8:1). In general, herbivores (rabbit fish) contain a higher proportion of ARA compared to omnivores and carnivores. This could be explained by the fact that ARA is abundant in macro and micro-algae, which is the main food of herbivores (Bell *et al.*, 1997; Bessonart *et al.*, 1999; Tocher *et al.*, 2000; Koven *et al.*, 2001; Bell and Sargent, 2003; Ogata *et al.*, 2004; Osako *et al.*, 2006; Suloma and Ogata, 2011; Monroig *et al.*, 2012; Jiarpinijnun *et al.*, 2017). In addition, siganid species contain the fatty acid desaturase and elongase enzymes in their body (Li *et al.*, 2010; Morais *et al.*, 2012). More importantly, siganids were reported to have Δ6/Δ5 Fads enzymatic activities essential for the biosynthesis of HUFAs using C18 PUFAs as starting substrate, which is unique amongst marine teleost (Xie *et al.*, 2016). Although most marine teleost cannot synthesize HUFAs themselves, it is possible to influence the FA metabolism of fish larvae, *e.g.* sea bream (*S. aurata*) (Seiliez *et al.*, 2003) and sea bass (*D. labrax*) (Vagner *et al.*, 2007) by a long-term nutritional conditioning on low HUFA compound diet (3 months). It is suggested that if the larvae were fed the low HUFA diet for long time, the Δ6 desaturase transcription of sea bass larvae was positively modulated by the HUFA-deprived diet (Vagner *et al.*, 2007). In our study, the rabbit fish were also fed with low HUFA content in the food (rotifers and *Artemia*) for 25 days (Table 6.4), and they are still healthy.

Table 6.4. LC-PUFAs (% of composition) of feed used in the hatchery

Fatty acids	*P. similis*	*B. rotundiformis*	*B. plicatilis*	*Artemia*	Compound diet
ARA	2.33	4.41	5.35	2.74	0.63
EPA	6.00	4.51	6.44	11.58	4.94
DHA	0.00	0.00	0.00	0.09	10.40
DHA/EPA	0.00	0.00	0.00	0.01	2.11

It is evident to conclude that the HUFA supplementation in the diet of rabbit fish is apparently less essential compared other marine teleosts, *e.g.* sea bass and sea

bream. The understanding of HUFA/PUFA synthesis by rabbit fish under aquaculture feeding schemes is important to optimise the larval and broodstock nutritional schemes.

Gut MC reflected the trophic level, life stage, host species, nutrition and environmental conditions (Sullam *et al.*, 2012; Navarrete *et al.*, 2013; Wong *et al.*, 2013; Giatsis *et al.*, 2014; Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b; Li *et al.*, 2014; Givens *et al.*, 2015; Liu *et al.*, 2016; Zarkasi *et al.*, 2016; Zha, 2017). It also plays important roles in the larval development, stress handling and disease resistance (O'Hara and Shanahan, 2006; Fjellheim *et al.*, 2007; Dhanasiri *et al.*, 2011). Factors that influence the gut MC of fish are summarized in Table 6.5.

Table 6.5. Factors influencing the gut MC of fish

Factors	References
Nutrition, dietary supplementation, probiotic addition	Ferguson *et al.* (2010); Ringø *et al.* (2012); Geraylou *et al.* (2013); Navarrete *et al.* (2013); Wong *et al.* (2013); Ingerslev *et al.* (2014a); Ingerslev *et al.* (2014b); Lobo *et al.* (2014); Merrifield and Carnevali (2014); Dawood *et al.* (2016); Zarkasi *et al.* (2016); Zhang *et al.* (2017); Zhou *et al.* (2017)
Environmental and ecological factors	Benson *et al.* (2010); Wong and Rawls (2012); Sullam *et al.* (2012); Yan *et al.* (2016); Dehler *et al.* (2017);
Host genetic factors & MC	Pérez *et al.* (2010); Benson *et al.* (2010); Givens *et al.* (2015)
Feeding habit, trophic level	Li *et al.* (2014); Liu *et al.* (2016)
Life stage & development	Stephens *et al.* (2016); Yan *et al.* (2016); Li *et al.* (2017c)
Physiological factors	Wong *et al.* (2013); Dehler *et al.* (2017)

The gut MC of rabbit fish larvae was also investigated both in wild and hatchery reared larvae by the Illumina NGS analysis. The NMDS analysis, based on the Bray-Curtis dissimilarity index showed that the gut MC of larvae between locations and hatchery, but not between years, was not only different in terms of diversity, but also different in terms of composition. The understanding the factors modulating the composition of the gut MC is important for development of fish larvae (van Kessel *et al.*, 2011). In this study, the differences in abiotic factors between locations, *e.g.* changes in water temperature, current direction and food quality, might impact the gut MC of rabbit fish in the wild. The fish gut MC from southern locations showed significant higher diversity than those from the north. During the sampling, the water temperature was measured and there is a positive correlation between changes in

water temperature and the relative distances of the gut MC among locations in this case. As fish are ectotherms, their body temperature always fluctuates following their environment; we need to understand how environmental temperature affects the gut MC. This study suggests that small environmental temperature differences influence to the fish gut MC. However, a causal link remains to be established. Other environmental parameters such as water current and difference in local food quality, as drivers of MC composition should be further investigated.

Next, the biotic factor (trophic level) also plays an important role in the genetic differences between the fish gut MC in the wild and hatchery. At the age of sampling (25 days old), while wild larvae are assumed to start consuming seaweed as food, the hatchery larvae are forced to feed on live food. This might impact the gut MC composition of the larvae, in term of cellulose-degrading bacteria. Low trophic level species usually contain high proportions of cellulose-degrading bacteria such as species belonging to the orders Clostridiales and Fusobacteriales. These orders were also predominant in the wild samples, but not in the hatchery. In the wild, the larvae feed predominantly on seaweed and aquatic plants. The presence of cellulose degrading bacteria, such as Clostridiales and Fusobacteriales, in the gut, strongly indicates that these larvae have already herbivorous feeding habit. These bacteria can convert cellulose into absorbable short chain fatty acids (Mohamed *et al.*, 2008; Thong-On *et al.*, 2012; Engel and Moran, 2013; Douglas, 2015; Amato, 2016; Liu *et al.*, 2016). These bacteria were not predominant in hatchery larvae, where the larvae were not fed any seaweed and aquatic plants in the diets. In addition, Saha *et al.* (2006); Zhou *et al.* (2009); Singh *et al.* (2010); Wu *et al.* (2012a); Wu *et al.* (2012b); Wu *et al.* (2013); Ye *et al.* (2014) and Hu *et al.* (2014) reported that different fish species or same fish species fed on different trophic levels and on dietary inputs had strong differences in the gut MC structure and diversity. Hence, the identification of gut MC does indicate that gut MC might be shaped by food in the wild. In the hatchery larvae, there was no proof to state that the gut MC of hatchery larvae was shaped through feeding, however, it might be shaped by the continuous addition of probiotics. In fact, the gut MC could be manipulated through the continuously input of bacteria strains (Burr *et al.*, 2005; Tinh *et al.*, 2008; Nayak, 2010). The effects of continuous feeding of probiotics on shaping the gut MC diversity and composition were confirmed in Siberian sturgeon (*A. baerii*) (Geraylou *et al.*, 2013), gilthead sea bream (*S. aurata*

L.) (Cerezuela *et al.*, 2013) and Atlantic salmon (*S. salar L.*) (Abid *et al.*, 2013; Zarkasi *et al.*, 2016). On the other hand, without the continuous input of probiotics, the gut MC manipulation of fish larvae was rather difficult, *e.g.* in Atlantic cod larvae (*G. morhua L.*) (Skjermo *et al.*, 2015). It is suggested that continuous or repeated addition of probiotics to the fish larvae is necessary for the manipulation of gut MC of fish larvae (Skjermo *et al.*, 2015).

In conclusion, this is the first report where a specific protocol for the large-scale culture of *P. similis* was developed. In turn this allowed for the reliable production of rabbit fish fingerlings. The new protocol can be the basis for further experiments on the production of fingerlings at larger scale. Despite the differences in the body DHA/EPA ratio of rabbit fish larvae caught in the wild and grown in the hatchery, the DHA/EPA ratio profile of hatchery larvae is still within the natural range. This suggests that rabbit fish larvae/fingerlings synthesise PUFA/HUFA themselves, an observation that is supported by recent publications and that merits further investigation. Hatchery larvae are growing well using a protocol that provides “continuous probiotic-enriched live food”. This practice together with the supply of live food shapes the MC in the gut of rabbit fish grown in the hatchery and makes the MC composition completely different from the MC present in wild larvae.

6.5. Future perspectives

As this is the first study pointing in the direction of reliable production of rabbit fish fingerlings, many production parameters can be investigated. The outcome of these studies could be used to further optimise rabbit fish larviculture.

6.5.1. Live food production

In relation to live food production it could be considered to replace live algae by dried algae (*N. oculata*) in *P. similis* culture (with probiotic addition) because the use of dried algae has many advantages: (i) lower price, (ii) longer shelf life, (iii) protozoan free and (iv) always available.

The LAB and *B. subtilis* strains used within works are selected based on their *in vitro* characters (e.g. based on their ability to growth at 20 $g.L^{-1}$ salinity) and the probiotic screening process has not been optimized exclusively for rabbit fish. Hence, further

screening and *in vivo* testing could be done to obtain the best probiotics for rabbit fish larviculture in the future.

6.5.2. Larviculture protocol and nutritional requirement of rabbit fish

This protocol should be the basis for further experiments on the larviculture of rabbit fish at commercial scale. Besides nutrition and probiotics, more parameters should be tested, e.g. like the impact of water management technique (recirculating systems and matured water), the influence of abiotic factors (*e.g.* continuous light, light intensity and light spectrum, but also oxygen supply systems to satisfy the oxygen requirement without causing too much turbulence, not to disturb the fragile larvae). Although not a topic of this research, broodstock nutrition and conditioning should be investigated, in order to improve the survival and performance of rabbit fish larvae.

Both literature data and this thesis suggest that rabbit fish can produce sufficient PUFA/HUFA on their own. It could be importance to establish in detail which PUFA precursors (if at all) are required and need to be supplemented through the feed to optimize PUFA/HUFA production in rabbit fish.

6.5.3. The gut MC investigation of rabbit fish larvae

The gut MC of the hatchery larvae using the current protocol is dominated by the supplemented probiotics and is lacking microbial species that might be essential to feed on algae in ponds. It could be important to verify if the gut MC needs to be conditioned to facilitate the transfer from the environmental conditions in the hatchery to the environmental conditions in the pond. Alternatively it could be that the dynamics of the shift in MC composition as larvae are being switched from carnivorous to herbivorous feeding behavior is so strong that MC conditioning is obsolete. Linked to this, the performance and growth of larvae from the hatchery should be verified while cultured in the grow-out system and compared with the performance of wild larvae.

APPENDIX

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- Teaching the subject of Diseases in Aquaculture to the third year student in aquaculture
- Doing research on aquaculture and aquatic diseases

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Integrated Management of Lagoon Activities Project (IMOLA) supported by FAO, Vietnam and Italian Cooperation

- Research poly-culture of giant tiger shrimp and mullet

2007- 2009: **Researcher**

Ghent University, Belgium

- The project on "Exercise to develop good management practices (GMP) for shrimp farming in Vietnam"

Honors & Awards

7/2017 **Global Minds Funds Award**, Ghent University, Belgium (Funded by VLIR-UOS)

9/2013 – 9/2017 **VLIR-IUC** Scholarship for Doctorate Study at Ghent Uni., Belgium

9/2010 – 9/2012 **VLIR-OUS** (Vlaamse Interuniversitaire Raad University Development Cooperation) Full Scholarship for Master Study at Ghent University – Belgium

2008 **Award of Inventive student** – Hue Uni. of Agriculture and Forestry

2008 **Award the Third Prize of the 'Vietnam - VIFOTEC Technological Creation for Student, 2008" Competition -** The National

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2004 - 2008 **Vietnamese Government Scholarship for undergraduate students** - Hue University of Agriculture and Forestry, Vietnam

Trainings and Conferences

2017 Poster - **LARVI 2017**, Ghent, Belgium.

2017 Oral - **World Aquaculture Society 2017**, Cape Town, South Africa

2016 Poster - **International Symposium on Fish Nutrition and Feeding**, Sun Valley, Idaho, USA

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Publications

Le, D.V., Nguyen, P.N., Dierckens, K., Nguyen, D.V., De Schryver, P., Hagiwara, A., Bossier, P., 2017. Growth performance of the very small rotifer *Proales similis* is more dependent on proliferating bacterial community than the bigger rotifer *Brachionus rotundiformis*. Aquaculture 476, 185-193.

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