SYSTEMIC AND MUCOSAL IMMUNE RESPONSE OF NILE TILAPIA BROODSTOCK TO MONOVALENT AND BIVALENT VACCINES AGAINST BACTERIA STREPTOCOCCUS AGALACTIAE AND AEROMONAS VERONII

by

QUENTIN ANDRES

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Aquaculture and Aquatic Resources Management

Examination Committee:	Dr. Krishna R.Salin (Chairperson) Dr. Ha Thanh Dong #1 Dr. YOUR COMMITTEE #2
Nationality:	French
Previous Degree:	Bachelor of Molecular Biology, Microbiology and Genetics University of Nice – Côte d'Azur Nice, France
Scholarship Donor:	None

Asian Institute of Technology
School of Environment, Resources and Development
Thailand
April 2022

AUTHOR'S DECLARATION

I, Quentin ANDRES, declare that the research work carried out for this thesis was in accordance with the regulations of the Asian Institute of Technology. The work presented in it are my own and has been generated by me as the result of my own original research, and if external sources were used, such sources have been cited. It is original and has not been submitted to any other institution to obtain another degree or qualification. This is a true copy of the thesis, including final revisions.

Date:

Name (in printed letters): QUENTIN ANDRES

Signature:

ACKNOWLEDGMENTS

Type your acknowledgments here. This section is typically reserved for personal and professional dedications. You may also acknowledge your donor or funder here or include copyright acknowledgements of journals whose articles you have significantly incorporated in this thesis. Maintain one blank 1.5 line spacing between paragraphs.

[one page, maximum]

ABSTRACT

Fighting bacterial infections inducing mass mortality in fish is a hot-topic research in the aquaculture industry in order to sustain its intensification. This research project aims to develop monovalent and bivalent vaccines against Group B streptococci (GBS) *S.agalactiae* and against Gram-positive (GP) *Aeromonas veronii* (*A.veronii*) for prophylaxis of *Nile tilapia* (*O.niloticus*) for which there is currently no vaccine available in Thailand. In the present research, FKV were produced by pathogen inactivation method: formalin 1% - 3 *%(v/v)* is added to the virulent pathogenic solution. The objective of the study is to characterize a part of the immune response elicited in *Nile tilapia* in response to vaccination. A total of $(\mathrm{n} = 400 juveniles)$ with an average mass of $(\mathrm{m} = X\mathrm{g} \pm X'\mathrm{g})$ were stocked into experimental ponds/aquariums prior to the start of the experiments. The population was then divided into 4 groups according to experimental design. After verification that the fish were free of any disease, they were acclimated for about a week. Meanwhile our 2 virulent pathogenic bacteria have been recovered and amplified on appropriate medium and were used for the vaccine production but also stored for challenge test. As a first step, a total of 4 treatments were administrated to the fish in order to monitor a possible immunization: 1 Control (Ct) (a vaccine with no immunizing properties) 3 formalin inactivated vaccines FKV containing either antigenic particles of *S.agalactiae* (monovalent Sa); *A.veronii* (monovalent Av); *S.agalactiae* + *A.veronii* (bivalent Sa+Av with a 1:1 ratio). In a second step, the fish immune response to vaccination (=immunogenicity and survivability) was determined by titrating agglutinating antibodies Ab but also with ELISA assay which is more reliable method. Indeed, isotype determination of Ab can allow to determine the concentrations of specific immunoglobulins M (IgM) and immunoglobulins T (IgT) for *S.agalactiae's* polysaccharides and *A.veronii*. In addition, gene transcription activity from before, during, and after the immunization (up to 70 days post-vaccination (dpv)) were determined by RT-PCR for IgM and IgT (active transcripts) corresponding to humoral immunity, and for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), nitric oxide synthetase (NOS) (respiratory burst activity of phagocytes, reactive oxygen species (ROS) producing) corresponding to innate immunity. As a final step, a challenge test in vivo was carried out on the fish juveniles with the two bacteria. Fish were injected with Control (Ct)+phosphate buffer saline (PBS) or were intra peritoneally injected a lethal dose of pathogen according to their prior vaccination.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

%(v/v)	Volume per volume (volume concentration of a solution)
A.veronii	*Aeromonas veronii*
O.niloticus*/*Nile tilapia	*Oreochromis niloticus*
S.agalactiae	*Streptococcus agalactiae*
Ab	antibodies
Ag	antigenic particles or Antigens
Av	Vaccine against *Aeromonas veronii*
CAT	catalase
Ct	Control
dpv	days post-vaccination
ELISA	enzyme-linked immunosorbent assay
FKV	formalin killed vaccines
GBS	Group B streptococci
GP	Gram-positive
GPx	glutathione peroxidase
HKV	Heat killed vaccines
HRP	horseradish peroxidase
IgM	immunoglobulins M
IgT	immunoglobulins T
IP/intra peritoneally injected	intra peritoneal injection
NOS	nitric oxide synthetase
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PRRs	pathogen recognition receptors
ROS	reactive oxygen species

RT-PCR	real-time reverse transcription polymerase chain reaction
Sa	Vaccine against *Streptococcus agalactiae*
Sa+Av	Bivalent vaccine against *S.agalactiae*+*A.veronii*
SOD	superoxide dismutase
SRT	sex-reveral therapy
TSB	Tryptic soy broth

Chapter 1

INTRODUCTION

1.1 Background of the Study

Previous outbreaks of *S.agalactiae* and *A.veronii* in *Nile tilapia*, Oreochromis niloticus, were reported in Japan, Taiwan, and the United States. *A.veronii* and *S.agalactiae* are widespread pathogens of *Nile tilapia* world-wide and induce mass mortality of the fish in a few days. The two bacterial isolates used in this research are from Thailand, where the bacteria are present and create substantial economic losses for Thai aquaculture industry.

1.2 Statement of the Problem

In order to sustain an intensified and resilient Cichlids' aquaculture, many research units and private companies around the world are studying immune responses of fish to viral and bacterial infections and are experimenting with vaccines development. There is currently no existing vaccine against the *S.agalactiae* and *A.veronii*. Oil-based FKV can be used as a prophylaxis treatment for aqua-cultured freshwater fish and this method is cost efficient and a viable alternative for the *Nile tilapia* that has not yet been developed in Thailand. A combined vaccination with a bivalent vaccine is the most cost-effective solution.

1.3 Hypothesis

Previous literature has shown the possibility to develop monovalent and multivalent vaccines for fish such as Asian seabass using whole inactivated pathogens. Assumption that Asian seabass and *Nile tilapia* share a similar physiology and similar dynamics in their response to antigenic particles or Antigens (Ag) support the possibility of making a vaccine for the *Nile tilapia*.

My second assumption is the feasibility of the FKV vaccine production using low-cost method with pathogen inactivation using a chemical: formalin with concentration of 1% - 3 *%(v/v)*, which would make the vaccines cost-efficient for farmers and medium sized farms.

1.4 Objectives of the research project

The objective of this research is to create new cost-effective vaccines in the Nile Tilapia against two fish pathogens *S.agalactiae* and *A.veronii*.

The three vaccines developed are in the table 1:

Table 1

A.veronii	Av
S.agalactiae	Sa
S.agalactiae + *A.veronii*	Sa+Av

The vaccines will be intra peritoneally injected (IP) in order to understand the elicited systemic and mucosal immune response of *Nile tilapia*. In addition, the efficiency of the monovalent formulations (Sa,Av) is compared versus the bivalent formulation (Sa+Av) will be compared with a challenge test.

1.5 Risks and limitations

1.5.1 Access to laboratory facilities

The experiments will be held and conducted at Asian Institute of Technology in the AARM department (stocking of fish and vaccination and challenge) and at SSRU laboratory facilities (ELISA, agglutination test, RT-PCR) and eventually at Mahidol Centex Shrimp.

1.5.2 Safety of experiments

Culture of the bacteria might not cause a risk to the wild fish and environment because all the experimentations will be made in closed and hermetic culture systems with compliance and respect of the strict biosecurity rules.

1.5.3 Risks in the schedule

The main challenges and risks could be due to any delays related fish keeping and fish culture or due to problems in the bacterial culture or vaccine preparation. It could eventually lead to a delay in the realization of the research project.

1.6 Organization of the research project

1.6.1 Project fundings

The project funding will come from the support of the AIT Innovative research fund.

1.6.2 Organization of the research project

I have divided my work in work packages.

I organize the rest of this research thesis as follows.

I have broken down the project work in several phases as shown in Figure 1.

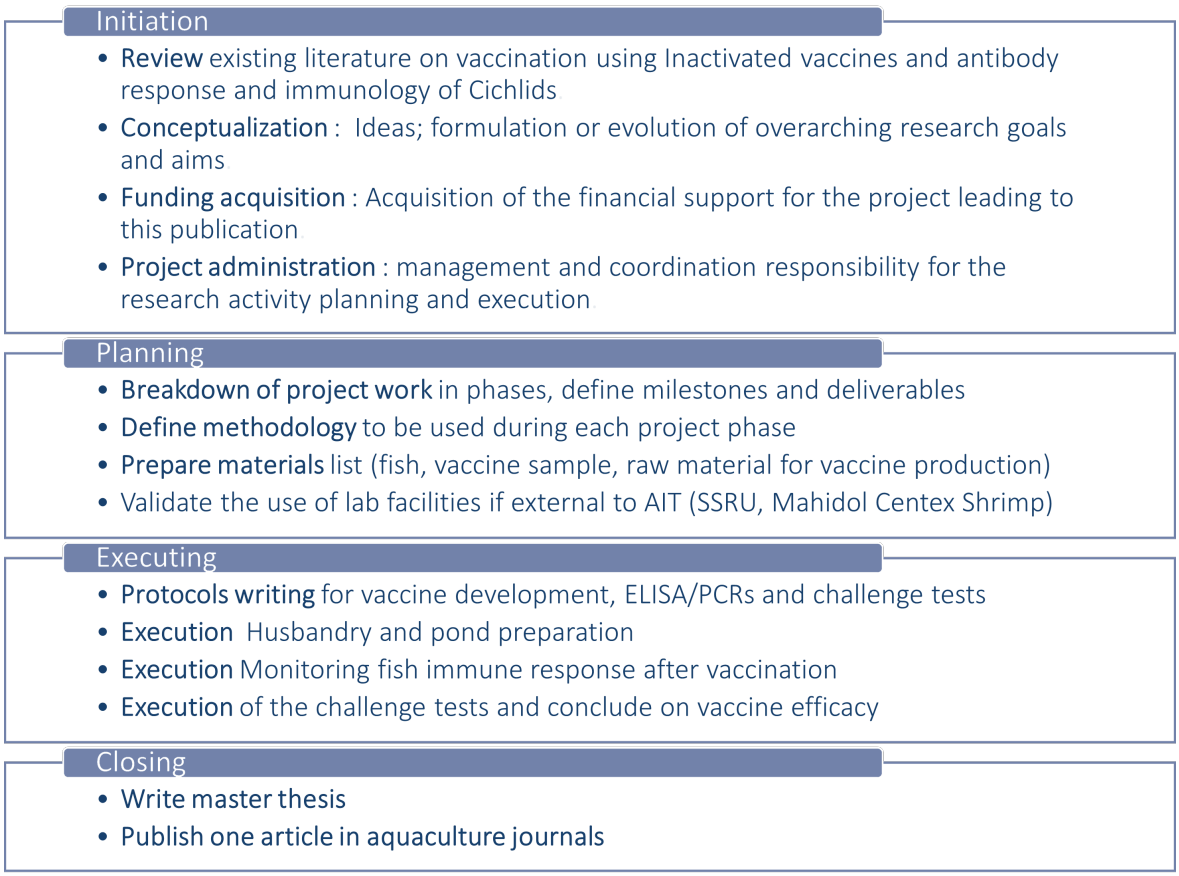
In Chapter 2, I describe the literature review.

In Chapter 3, I propose my methodology for the experimental design, for the pond preparation, the fish feeding regime. But also for bacterial culture, vaccination, fish mucus swabs and blood sampling. For antibody agglutination titration, ELISA, RT-PCR and finally for the Challenge tests.

In Chapter 4, I present the experimental results.

Finally, in Chapter 5, I conclude my thesis.

Figure 1: Steps of the research project



Chapter 2

RELEVANT LITERATURE

TO BE DONE LATER This chapter will provide a review of the relevant literature for which my project is based on.

2.1 Relevant literature on *S.agalactiae* and *A.veronii* and their infections in Nile tilapia

2.1.1 *S.agalactiae* pathogenesis

Some texts

2.1.2 *A.veronii* pathogenesis

2.2 Relevant literature on whole pathogen inactivated vaccines

Some texts

2.2.1 Formalin killed vaccines (FKV)

Some texts

2.2.2 Heat killed vaccines (HKV)

Some texts

2.3 Relevant literature on systemic and mucosal fish immunology: Innate immunity

Some texts

2.3.1 Recognition of self and non-self, PAMPs and PRRs

Some texts

2.3.2 The complement system

Some texts

2.3.3 Neutrophils

Some texts

2.3.4 Phagocytosis

Some texts

2.3.5 Oxygenic respiratory burst

Some texts

2.4 Relevant literature on systemic and mucosal fish immunology: Specific immunity

Some texts

2.4.1 Immunoglobulins in fish

Some texts

2.4.2 T cells and B cells

Some texts

2.5 Relevant literature on antibody agglutination titration, ELISA (humoral immune system)

Some texts

2.5.1 Antibody agglutination test

Some texts

2.5.2 Enzyme-linked immunosorbent assay (ELISA)

Some texts

2.6 Relevant literature on leucocytes count and quantification of respiratory burst activation (innate immune system)

Some texts

2.6.1 Leucocyte cell count and haemodynamic parameters in fish.

Some texts

2.6.2 Assays for the mesurement of the respiratory burst (reactive oxygen species ROS) and phagocytosis activity.

Some texts

2.7 Chapter Summary

Some texts

Chapter 3

METHODOLOGY AND RAW MATERIAL

Some intro..

3.1 Project steps overview

I will describe in the next paragraphs the methodology I will use for each of the steps described in Figure 3. TO BE CONTINUED

3.2 Experimental design and statistical power analysis

Table 2.1

Informations about animals and their origins

Fish population $(\mathrm{n} = 400 juveniles)$.
Species = *O.niloticus*.
Age = *juveniles*.
Average mass of $(\mathrm{m} = X\mathrm{g} \pm X'\mathrm{g})$.
Secondary sexual gender: **100% underwent sex-reveral therapy (SRT)**.
Origin = **Thailand**, Asian institute of technology.
Strain = **Chitralada 4**.
Pathogen free = **required**.

Detection of diseases (procedure): Select **6-10** individuals at random, kill them, culture brain and head kidney on Tryptic soy broth (TSB) for detection of pathogens (*A.veronii* and *S.agalactiae*).

How many groups for the vaccination? Create **4** groups/subsets of approx. **100-150** individuals. **4** ponds/aquariums for **10** days(Acclimatation) + **70** days(post vaccination) = **80** days.

Methodology for flood and mucus sampling: Select not less than **8** fish per group for **blood** and **mucus** swab every week.

Challenge test: Separate the fish in 2 sub-groups for Ct, Av **monovalent**, Sa **monovalent**. In 3 sub-groups for Sa+Av **bivalent**, as indicated below:

Table 2.2 Summary of the different groups getting vaccinated.

Table 2.2

Summary of the different groups getting vaccinated

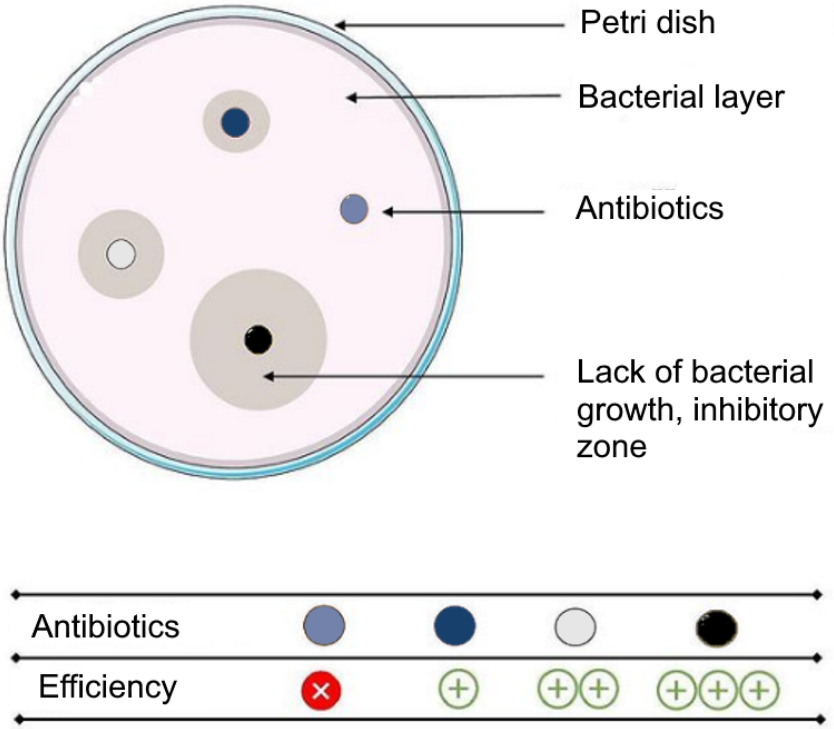
Control (sham vaccinated) challenged with *S.agalactiae* = **33** fish.
Control (sham vaccinated) challenged with *A.veronii* = **33** fish.
Control (sham vaccinated) challenged with PBS = **33** fish.
Sa **monovalent** challenged with *S.agalactiae* = **50** fish.
Sa **monovalent** challenged with PBS = **50** fish.
Av **monovalent** challenged with *A.veronii* = **50** fish.
Av **monovalent** challenged with PBS = **50** fish.
Sa+Av **bivalent** challenged with *S.agalactiae* = **33** fish.
Sa+Av **bivalent** challenged with *A.veronii* = **33** fish.
Sa+Av **bivalent** challenged with PBS = **33** fish.

Statistical power analysis: It is possible to create an artificial population of each group composed of 20 000 sampling with replacement and use the Central limit theorem to deduce the Standard error of the means and infer our vaccine efficiency for all the juveniles Chitralada 4 in the world (main population) with 95% confidence using an approximation of the Standard deviation.

3.3 Methodology for pond preparation and fish stocking

3.4 Methodology for bacterial culture and bacterial preparation

Figure 3: Schema of an antibiogram



3.4.1 For *S.agalactiae*

Antibiogram *S.agalactiae*:

3.4.2 For *A.veronii*

Antibiogram *A.veronii*:

3.5 Methodology for preparation of formalin killed vaccines (FKVs)

Volume to inject 100µL IP/$fish$

3.6 Methodology for vaccine administration in fish and monitoring of fish health during bacterial challenge test

3.7 Methodology for fish sera and mucus extraction

3.8 Methodology for ELISA assays for specific IgM, IgT titrations

ELISA is an enzyme-linked immunosorbent assay "for the presence of antibodies, antigens", proteins and glycoproteins in biological samples. ELISA technique is widely used for rapid diagnostic tests such as the diagnosis of HIV infection, pregnancy tests or the detection of food allergens.

The principle of this technique is based on the use of an enzyme conjugated to an antibody which by reacting with a colorless substrate gives a colored reaction product and which is therefore detectable. This is called a chromogenic substrate. Different enzymes are used for ELISA tests including alkaline phosphatase, horseradish peroxidase (HRP) or beta-galactosidase.

The ELISA assay will give information on the Ab titer of the fish, IgM and IgT respectively will be targeted in the assay.

3.9 Methodology for RT-PCR

The reference genes for the RT-PCR of *O.niloticus* are genes ubiquitously and constitutively expressed in the animal. They are also well known as "house-keeping" genes/mRNAs. (see below).

Figure 4: Reference genes used in RT-PCR technique for Tilapia
*Credits:*http://dx.doi.org/10.1016/j.gene.2013.06.013

Table 3
Average Ct values during RT-qPCR of the candidate reference genes in selected tissues before and after *S. agalactiae* infection, *S. iniae* infection, and PBS injection.

	Injection	B2M	UBCE	EF1A	GADPH	TUBA	ACTB	18S rRNA
Liver	*S. agalactiae*	17.87 ± 1.85	28.43 ± 0.64	18.09 ± 0.47	23.84 ± 1.43	25.55 ± 0.78	19.78 ± 0.55	12.32 ± 0.51
	S. iniae	18.33 ± 2.96	28.77 ± 1.51	17.78 ± 1.63	23.24 ± 2.45	24.94 ± 2.10	19.98 ± 1.93	12.64 ± 0.92
	PBS	17.31 ± 0.82	28.69 ± 0.78	17.13 ± 0.62	22.68 ± 1.86	24.12 ± 1.82	19.00 ± 0.92	12.72 ± 0.70
Intestine	*S. agalactiae*	15.74 ± 0.76	26.9 ± 0.38	17.86 ± 0.79	21.41 ± 0.76	23.19 ± 0.49	18.07 ± 0.74	11.98 ± 0.24
	S. iniae	15.60 ± 1.37	27.35 ± 1.16	17.48 ± 1.12	22.08 ± 2.27	23.77 ± 1.17	19.00 ± 1.79	12.07 ± 0.29
	PBS	15.14 ± 0.34	26.20 ± 0.48	16.64 ± 0.93	20.48 ± 0.85	22.57 ± 1.02	16.93 ± 0.92	11.97 ± 0.22
Kidney	*S. agalactiae*	14.53 ± 0.67	26.11 ± 0.82	18.35 ± 1.28	17.95 ± 0.95	23.02 ± 0.43	17.11 ± 0.89	13.40 ± 0.52
	S. iniae	14.72 ± 1.08	25.36 ± 0.54	18.17 ± 0.72	18.64 ± 0.75	22.05 ± 1.27	15.81 ± 1.56	13.28 ± 0.99
	PBS	13.73 ± 0.30	25.82 ± 0.40	18.07 ± 0.87	18.57 ± 1.12	22.71 ± 0.30	16.66 ± 0.78	13.51 ± 0.74
Heart	*S. agalactiae*	17.13 ± 0.46	27.44 ± 0.71	18.15 ± 0.66	19.26 ± 1.01	20.44 ± 0.93	13.51 ± 0.77	12.60 ± 0.49
	S. iniae	15.78 ± 1.86	26.39 ± 1.58	16.94 ± 1.18	18.82 ± 1.47	19.55 ± 1.13	12.70 ± 1.04	12.21 ± 0.79
	PBS	16.56 ± 0.38	27.24 ± 0.18	17.62 ± 0.60	19.38 ± 0.40	20.64 ± 0.71	14.33 ± 1.98	12.03 ± 0.99
Brain	*S. agalactiae*	19.93 ± 0.92	25.73 ± 0.23	18.78 ± 0.35	17.14 ± 0.30	20.62 ± 0.29	16.64 ± 0.22	12.35 ± 0.77
	S. iniae	19.69 ± 0.93	25.75 ± 0.18	18.85 ± 0.45	17.31 ± 0.34	20.47 ± 0.35	16.67 ± 0.20	12.83 ± 0.55
	PBS	19.34 ± 0.43	25.40 ± 0.31	17.93 ± 0.68	16.89 ± 0.33	20.68 ± 0.33	16.29 ± 0.45	12.76 ± 0.88
Spleen	*S. agalactiae*	16.15 ± 1.17	26.08 ± 0.47	17.28 ± 0.61	18.22 ± 1.42	21.42 ± 0.52	15.72 ± 0.61	11.54 ± 0.41
	S. iniae	15.79 ± 0.99	26.29 ± 0.36	17.24 ± 0.70	17.95 ± 0.90	21.08 ± 0.33	15.51 ± 0.55	11.17 ± 0.56
	PBS	15.38 ± 0.54	25.76 ± 0.31	16.90 ± 0.34	17.95 ± 1.05	20.75 ± 0.78	14.78 ± 0.52	10.98 ± 0.55
Muscle	*S. agalactiae*	21.44 ± 1.45	30.10 ± 1.20	21.29 ± 0.81	23.85 ± 1.12	25.03 ± 0.89	18.84 ± 0.47	14.37 ± 0.33
	S. iniae	21.86 ± 0.65	30.07 ± 1.09	21.32 ± 0.74	24.25 ± 1.06	25.61 ± 0.96	18.49 ± 0.42	14.55 ± 0.44
	PBS	21.10 ± 0.86	29.90 ± 1.36	20.76 ± 1.14	24.09 ± 1.30	25.13 ± 1.09	18.70 ± 0.53	14.47 ± 0.39

3.10 Methodology for antibody agglutination titration

3.11 Methodology for data curation and result analysis

ANOVA 1 way, + post hoc test tukey with signif not more than 0.05, Rstudio

3.12 List of raw material

3.12.1 Price of raw material

3.13 Chapter Summary

Chapter 4

Experimental Results

Some intro..

4.1 Section Name in Experimental Results

Chapter 5

Conclusion and Recommendations

Some text..

5.1 Conclusion

Text..

5.2 Recommendations

Text..

Bibliography

yamato92hmmYamato, J., Ohya, J. Ishii, K. 1992. Recognizing human action in time-sequential images using hidden Markov model. International Conference on Computer Vision and Pattern Recognition (CVPR) (379–385). [p]

APPENDIX A : useful documents

Planning august 2021 - march 2022

PLANNING		AUG			SEP				OCT					NOV			
		Aug-09			Aug-30						Oct-04			Nov-01			
WP	Tasks/Dates	W32	W33	W34	W35	W36	W37	W38	W39	W40	W41	W42	W43	W44	W45	W46	W47
	WP1 - Master thesis prep																
T1.1	WP1-T1 - proposal	T1.1	T1.1	T1.1													
T1.2	WP1-T2 - first draft																
T1.3	WP1-T3 - final draft																
T1.4	WP1-T4 - regular check	T1.4			T1.4			T1.4			T1.4			T1.4			T1.4
	WP2 - Pond, fish and husbandry																
T2.1	WP2- prepare pond, stock breeders, select feeding regime				T2.1	T2.1	T2.1	T2.1	T2.1	T2.1	T2.1						
T2.2	WP2- Feed the offspring and raise 400 juveniles.						T2.2	T2.2	T2.2	T2.2	T2.2						
T2.3	WP2 - Quality control, fish free of diseases										T2.3						
T2.4	WP2- Prepare 4 medium experimental ponds for Ct, Sa, Av, SaAv											T2.4					
T2.5	WP2- Acclimatation 10-14days											T2.5	T2.5				
T2.6	WP2- Prepare at least 9 small ponds for the challenge test																
T2.7	WP2- Clean the ponds at the end of the experiment																
	WP3 - Bacterial preparation, vaccine preparation																
T3.1	WP3- Recovery of the two strains											T3.1	T3.1				
T3.2	WP3- Amplification and culture for challenge test												T3.2	T3.2	T3.2	T3.2	T3.2
T3.3	WP3- Production of vaccine												T3.3				
T3.4	WP3- Stockage of vaccine												T3.4	T3.4	T3.4	T3.4	T3.4
T3.5	WP3-Stockage of bacteria for challenge test																
T3.6	WP3-Verify the concentration																
	WP4 - Immunization, fish swab, challenge test																
T4.1	WP4- Fish Swab before immunization-acclimatation										T4.1						
T4.2	WP4- Immunization														T4.2		
T4.3	WP4- Fish swabs weekly (keep at cold temperature)													T4.3	T4.3	T4.3	T4.3
T4.4	WP4- Challenge test with the two strains																
T4.5	WP4- Draw results of the challenge test																
T4.6	WP4- Discussions																
	WP5 - ELISA, PCR, Agglutination test																
T5.1	WP5- ELISA for each group of samples for IgM and IgT																
T5.2	WP5- PCR of key immune genes																
T5.3	WP5- Agglutination test																
T5.4	WP5- Results and interpretations																
T5.5	WP5- Conclusions																
T5.6	WP5- Recommendations																
	WP6 - Ethics requirements																
T6.1	WP6 - Ethics requirement 1: Animal welfare during experimentation																
T6.2	WP6 - Ethics requirement 2: Approve use of fish for the study by Animal Care and Use Committee																

PLANNING (continued)		DEC				JAN					FEB				MAR		
		Dec-06				Dec-27					Jan-31				Feb-28		
WP	Tasks/Dates	W48	W49	W50	W51	W52	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11
	WP1 - Master thesis prep																
T1.1	WP1-T1 - proposal																
T1.2	WP1-T2 - first draft																
T1.3	WP1-T3 - final draft																
T1.4	WP1-T4 - regular check		T1.4		T1.4		T1.4			T1.4			T1.4			T1.4	
	WP2 - Pond, fish and husbandry																
T2.1	WP2- prepare pond, stock breeders, select feeding regime																
T2.2	WP2- Feed the offspring and raise 400 juveniles.																
T2.3	WP2 - Quality control, fish free of diseases																
T2.4	WP2- Prepare 4 medium experimental ponds for Ct, Sa, Av, SaAv																
T2.5	WP2- Acclimatation 10-14days																
T2.6	WP2- Prepare at least 9 small ponds for the challenge test																
T2.7	WP2- Clean the ponds at the end of the experiment																
	WP3 - Bacterial preparation, vaccine preparation																
T3.1	WP3- Recovery of the two strains																
T3.2	WP3- Amplification and culture for challenge test																
T3.3	WP3- Production of vaccine																
T3.4	WP3- Stockage of vaccine																
T3.5	WP3-Stockage of bacteria for challenge test																
T3.6	WP3-Verify the concentration																
	WP4 - Immunization, fish swab, challenge test																
T4.1	WP4- Fish Swab before immunization-acclimatation																
T4.2	WP4- Immunization																
T4.3	WP4- Fish swabs weekly (keep at cold temperature)																
T4.4	WP4- Challenge test with the two strains																
T4.5	WP4- Draw results of the challenge test																
T4.6	WP4- Discussions																
	WP5 - ELISA, PCR, Agglutination test																
T5.1	WP5- ELISA for each group of samples for IgM and IgT																
T5.2	WP5- PCR of key immune genes																
T5.3	WP5- Agglutination test																
T5.4	WP5- Results and interpretations																
T5.5	WP5- Conclusions																
T5.6	WP5- Recommendations																
	WP6 - Ethics requirements																
T6.1	WP6 - Ethics requirement 1: Animal welfare during experimentation																
T6.2	WP6 - Ethics requirement 2: Approve use of fish for the study by Animal Care and Use Committee																

APPENDIX B

Section Name

Some text ..

VITA

Section Name

Some text ..