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The Potential Roles of Gut Microbiome in Modulating the Immune Response of Asian Redtail Catfish (*Hemibagrus nemurus*) Vaccinated with *Aeromonas hydrophila*

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ABSTRACT

The research aims are to observe the effect of vaccination in microbial profiles and gut microbiome composition. The treatments were as follows: the fishes were injected with PBS and challenged (A); the fishes were injected with freeze–dried vaccine dissolved in 100 ml 0.85% NaCl and challenged (B); the fishes were injected with freeze–dried vaccine dissolved in 50 ml 0.85% NaCl and challenged (C), and the fishes were injected with liquid vaccine and challenged (D). Microbiome composition measurements were carried out on the 21st–day post–vaccination and the 7th day after the challenge test. Fish intestine samples from three replications were tested by Next Generation Sequencing (NGS). Two significant phyla were identified from all treatments, namely *Proteobacteria* and *Firmicutes*. *Cetobacterium*, *Candidatus Bacilloplasma*, and *Clostridium sensu stricto* were the genera classified as good bacteria in vaccinated fish. It can be concluded that vaccination can increase the diversity of the gut microbiome, especially bacteria beneficial to the fish host. Chitosan as a coating antigen in freeze–dried vaccine increases gut microbiome's number and diversity better than a liquid vaccine.

1. Introduction

The ecosystem of the fish gut is very complex. There are 10^7 to 10^{11} bacteria g^{-1} in the fish gut as inhabitants of fish intestines (Nayak 2010). Llewellyn *et al.* (2014) said that aerobic, facultatively anaerobic, and obligate anaerobic bacteria are the main bacteria in fish gut. The mutually beneficial relationship between the gut microbiome and its host is a symbiotic mutualism (Brown *et al.* 2013). The development and function of the immune system are played by the whole microbiome or by their respective roles (Jamieson 2015). Vaccines are modulated by the gut microbiome, which plays a role in the immune system, but the mechanism for the emergence of this immunomodulatory effect is not yet known (Ferreira *et al.* 2010; Wu and Wu 2012). According to Clarke *et al.* 2010 and Wu *et al.* 2010 immunomodulatory effect is obtained through factors that dissolve and are secreted into the blood circulation or through lymphocytes in the lamina propria of the gut.

The gut microbiome produces factors such as metabolic products. It has many roles in intestinal integrity, stimulating cytokine production to increase pro–inflammatory or anti–inflammatory activity and supplying vitamins and short–chain fatty acids (SCFAs) to the host (Hooper *et al.* 2012). SCFA, produced by the gut microbiome through a fermentation process, functions as a modulator of IECs and plays a role in the development of leukocytes (Corrêa–Oliveira *et al.* 2016). Gut microbiome such as genus clostridium is the primary production of SCFA and is experimentally associated with potent anti–inflammatory activity. In contrast, the genus Lactobacillus is categorized as a low producer of SCFA but concentrated lactic acid. The SCFA butyric acid is a secondary production of lactic acid (Wullt *et al.* 2007).

Some researchers, such as Jamieson (2015) and Zimmermann and Curtis (2018), argue that the microbiome influences vaccination, causing an immune response. It was also emphasized by Lynn

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et al. (2018), especially in applying injection and oral vaccination. Factors that play a role in response to vaccination are the characteristics of the vaccine, the dose, the route of immunization, and the vaccination schedule. Vaccine formulations such as application via injection (parenteral), oral or immersion (mucosa), presence or absence of adjuvants and immunomodulators, antigen properties such as intact cells, purified proteins, polysaccharides, and nucleic acids) influence the host's immune response and the strategy of vaccinations such as homologous and heterologous prime–boost and the interval between doses. (Harandi and Medaglini 2010; Fiorino *et al.* 2013; Ciabattini *et al.* 2013, 2015, 2016, 2018; Cunningham *et al.* 2016; Dacoba *et al.* 2017; Handel *et al.* 2018). Based on Jamieson (2015) and Zimmermann and Curtis (2018), the immune response that occurs due to vaccination is influenced by the condition of microbiomes in fish's gut, so that these research aims are to identify the effects of the vaccination of liquid and freeze–dried vaccines in microbial profiles and gut microbiome composition.

2. Materials and Methods

2.1. Fish Samples and Bacteria

The test fish used was *Hemibagrus nemurus* (25±1.5 g). Fishes were transported from Bogor and Depok West Java area to Fish Health Laboratory, Depok West Java, using a plastic bag provided with O_2. All fishes that were used have passed the acclimatization period of 14 days prior treatment test. *Aeromonas hydrophila* AHL0905–2 was used as a vaccine master seed, and it was cultured in Tryptic Soy Agar (TSA) before vaccine production.

2.2. Whole–Cell Vaccine Preparation

The vaccine was prepared using the dry planting method in agar medium. *A. hydrophila* as the master seed was injected into fishes to produce Koch's postulates. *A. hydrophila* bacteria resulting from Koch's postulates were inoculated in TSA media, incubated for ±24 hours at 27°C. The growing bacterial colonies were collected using a loop needle and put in a 150 ml volume bottle containing 100 ml of sterile saline solution (0.845% NaCl). Furthermore, the bacterial suspension weres inactivated by adding 3% (v/v) NBF (Neutral Buffer Formalin) through stirring for ±6 hours. The concentration of vaccine stock suspensions was measured using a spectrophotometer at a wavelength of 660 nm, and platting was performed to determine the cell forming unit (CFU). The vaccine stock obtained was then stored in a freezer at –60°C for ±24 hours. The frozen vaccine stock was dried in a vacuum dryer at –100°C (CoolSafe™ freeze dryer) for 36–48 hours. After drying, the vaccine was stored at 4°C, while for long–term storage, the vaccine was stored at –20°C.

2.3. Preparation of Chitosan Coated Vaccine

The liquid vaccine was taken 30 ml, and chitosan was added as a coating material with a predetermined concentration based on the modification of coating material for probiotics carried out by Cock and Castillo (2013) and Kanmani *et al.* (2011). The vaccine was then homogenized for 5 minutes and put in the freezer for 24 hours to obtain a frozen vaccine as a requirement before the freeze–drying process was carried out. The freeze–drying process used the Coolsafe Scanvac apparatus from Chemoscience Pte Ltd. by starting the engine until the temperature indicator reached –100°C. The sample was inserted, and a vacuum was applied to remove the water content in the sample. The freeze–drying process was carried out for three days until the sample changed shape to freeze dry.

2.4. Vaccine Efficacy Test and Challenge Test

Freeze–dried vaccine efficacy testing was carried out on fish with three replications and control of PBS injection. The freeze–dried vaccine was diluted using 100 ml and 50 ml sterile saline solution (NaCl 0.845%) until the suspension was dissolved. Fishes were cultured in 400 L of water, with a density of 100 fishes/fiber tank and triplicate-fish vaccination through intraperitoneal injection at a dose of 0.1 ml kg^{-1} of body weight. The Challenge test was carried out 14 days post–vaccination. Challenge test used virulent *A. hydrophila* at a dose of LD_{50} (1 x 10^7 CFU ml^{-1}). The fish were observed for microbiome composition at the 21st post–vaccination and 7th–day post–challenge test. The treatments were as follows: A. non–vaccinated fish (NoVac); B. freeze–dried vaccinated fish (VKB50); C. liquid vaccinated fish (LiqVac); D. post–challenge freeze–dried vaccinated fish (PCVKB50); E. post–challenge liquid vaccinated fish (PCLiqVac). The Ethical Experimentation Committee of Research Agency and Human Resources, The Ministry of Marine Affairs, and Fisheries Indonesia approved these experiments.

2.5. Composition of The Gut Microbiome

Microbiome composition measurements were carried out on the 21st post–vaccination and the 28th day after the challenge test. Fish intestine samples from three replications were tested by Next Generation Sequencing (NGS). Anesthetic solution clove oil was used for fish anesthetic. Before fish dissection, the fish's outer surface was disinfected using 70% ethanol and rinsed with sterile distilled water three times. Then the entire intestine was removed from the abdominal cavity, and 5 g fish intestine of each replication was taken, weighed using a digital scale, collected in a sterile plastic tube, then immediately stored at –80°C until further processing. Samples from after vaccination and post–challenge tests treatments were sent to the NovogeneAIT Laboratory for NGS testing which included DNA extraction process, PCR amplification and purification, library preparation, and quality control check. The results of genomic DNA extraction from fish intestines were sequenced using the CTAB/SDS method. The concentration and purity were checked using 1% agarose gel diluted first to one ng/µl with sterile water. Specific primers were used to amplify 16S rRNA/18SrRNA/ITS areas run using the Phusion® High–Fidelity PCR Master Mix (Biolab New England). The specific primers for example V3 and V6 regions (Untergasser *et al.* 2012), the designed primers were used together with a universal primer pair, targeting the V4 hypervariable region of the 16S rRNA gene, amplified with the modified versions (Apprill *et al.* 2015).

The quantification and qualification of PCR products are carried out using green SYBR with the same volume as 1x loading buffer. PCR products were electrophoresed on 2% agarose gel. The bands that appeared between 400–450 bp were subjected to further analysis. The mixing and purification of PCR products were carried out using Qiagen Gel Extraction Kit (Qiagen, Germany) based on the equilibrium ratio of yield, specificity, and precision of the PCR product.

2.6. Bacterial Community Analyses

TruSeq® DNA PCR–Free Sample Preparation Kit (Illumina, USA) was used for library and sequencing and then measured using the Qubit @ 2.0 Fluorometer (Thermo Scientific) system and Agilent Bioanalyzer 2100. Library order was carried out using the Illumina HiSeq2500 platform with a size of 250 bp.

3. Results

Vaccination causes the appearance of antibodies in fish. Antibodies were formed in the first week to the second week, from low to high titer concentrations but decreased in the third–week post–vaccination. Freeze–dried vaccine provided consistently between week two and week three. Meanwhile, the liquid vaccine showed an increased antibody titer in the second week but decreased dramatically in the third week. In control, antibody titer was very low.

The highest microbiome in the digestive tract is the VKB50 treatment group, compared to the LigVac treatment group (this is in the second largest position) and the NoVac group. Whilst, PCLiqVac treatment group had a higher abundance of microbiome than PCVKB50 treatment group (Figure 1).

Five samples was analyzed and yielding 102,664 DNA sequences and 4815 identified species. Figure 1 also presents the number of OTUs and sequences for NoVac, VKB50, LiqVac, PC VKB50, and PCLiqVac. VKB50 treatment group produced the highest number of OTUs and sequences.

In Figure 2 presented the Venn diagram of the microbiome in the digestive tract of all treatment group. Unique OTU is owned by all treatments group namely 56 for NoVac group, 60 and 63 OTU unique for VKB50 group and LiqVac group, 37 and 51 for PCVKB50 and PCLiqVaq group. Meanwhile, OTUs shared by all treatments are 539. The VKB50 treatment of the Shannon and Simpson species diversity index was higher, namely 5.81 and 0.95, compared to the LiqVactreatment group and NoVac group. Meanwhile, the LiqVac group had a higher abundance of microbiome than the NoVac group. It is indicated by the Shannon and Simpson species diversity index, which are 5.29 and 0.91 for the LiqVac, while the NoVac groups were 3.82 and 0.74 (Table 1).

The heat–map analysis showed the entire genus present in all treatments that appear to be different between NoVac, vaccinated (VKB50 and LiqVac), and challenged groups (PCVKB50 and PCLiqVac) (Figure 3). In the digestive tract of fish, eight microbiomes appeared in the heat map, namely *Actinobacteria, Bacteriodetes, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria Tenericutes*, and *Verrucomicrobial* groups. Each treatment shows a different group, from orange to red on the heat–map. *Terrisporobacter, Cellulosilyticum,*

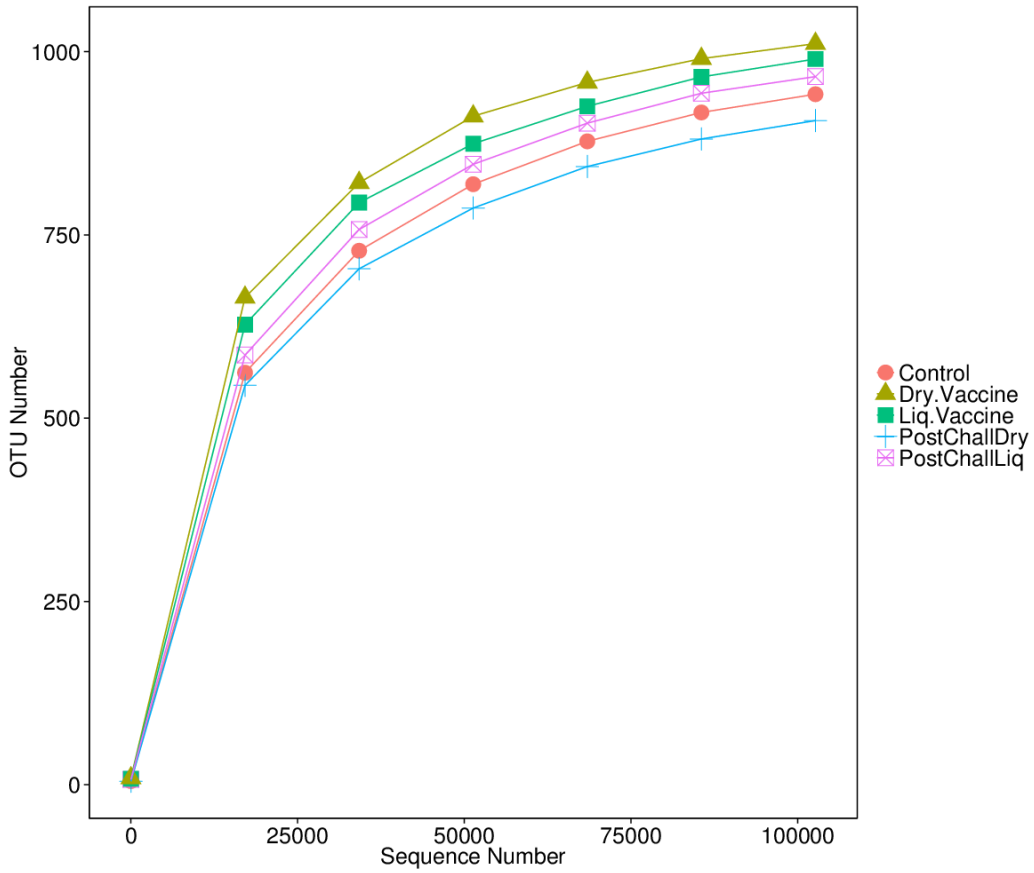

Figure 1. Gut microbiota abundance in the digestive tract

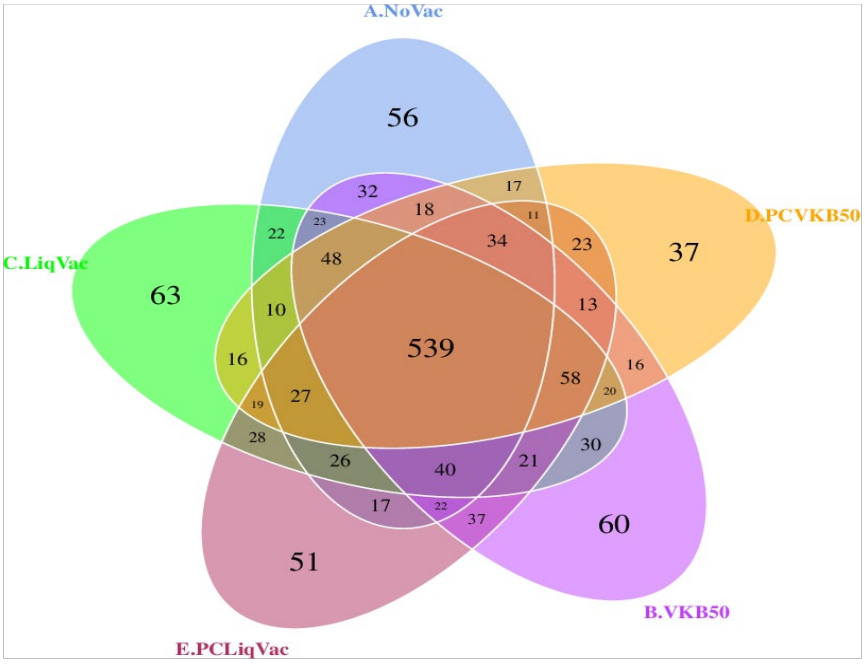

Figure 2. Venn diagram of the microbiota in the digestive tract

and *Candidatus arthromitus* dominated NoVac group. Meanwhile, the LiqVac group was dominated by *Bacillus, Acinetobacter, Oryza sativa japonica, Streptomyces, Plesiomonas, Clostridium sensu stricto,* and *Romboutsia*. The VKB50 group was dominated by *Ruminococcocoaceae, Acidisphaeria, Cetobacterium, Demequina, Alistipes, Turicibacter, Acidothermus,* and *Candidatus bacilloplasma.*

The phyla composition of each treatment, dominated by red color, namely *Proteobacteria* and blue color, namely the *Firmicutes* group, although the numbers differ in each treatment. In the VKB50 treatment group, Phyla *Fusobacteria* showed a greater relative abundance than other treatments and decreased slightly after the challenge test (Figure 4).

Three vertexes represent three treatments, no vaccination (NoVac) group, liquid vaccine group (LiqVac) and freeze–dried vaccine dissolved in 50 ml 0.85% NaCl group (VKB50). Figure 5 showed red and grey circles represent dominant taxa and the diameter size of circles related to the relative abundance. All treatment has red circle of (*Firmicutes* phylum) because it was located in the center of Ternaryplot. The green and purple circles (*Tenericutes* and *Fusobacteria* phyla) close to VKB50 treatment group which has higher abundance of this phylum.

Figure 6 shows the genus composition of all treatments. On VKB50 treatment, the microbial genus appeared to be more varied in type, followed by the liquid vaccine treatment. The genus *Cetobacterium*

Table 1. Species diversity index of Shannon and Simpson

Treament	Observed species	Shannon	Simpson
A. NoVac	942	3.819	0.736
B. VKB50	1011	5.807	0.952
C. LiqVac	990	5.291	0.914
D. PCVKB50	906	3.538	0.613
E. PCLiqVac	966	4.708	0.850

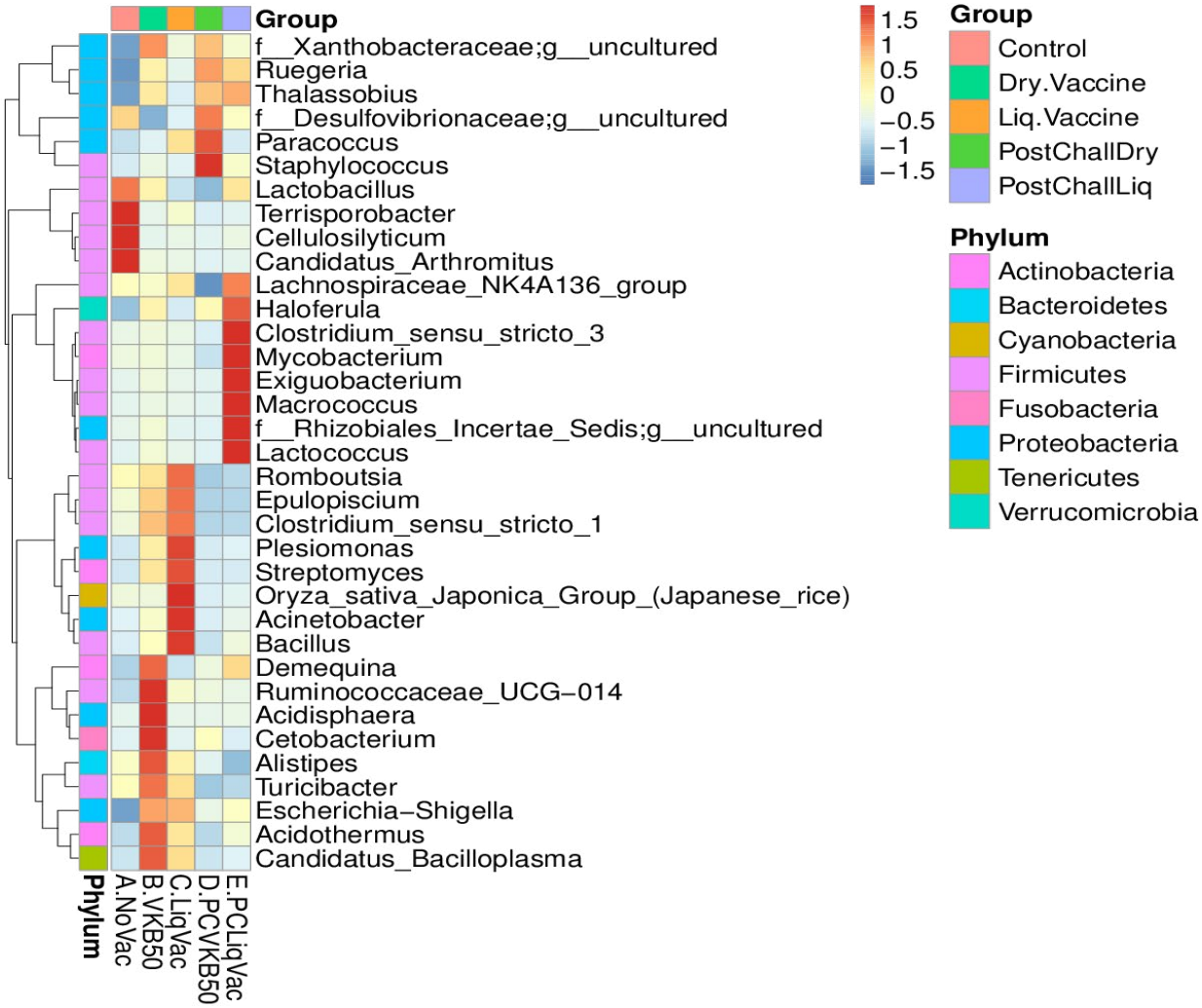

Figure 3. Heat-map

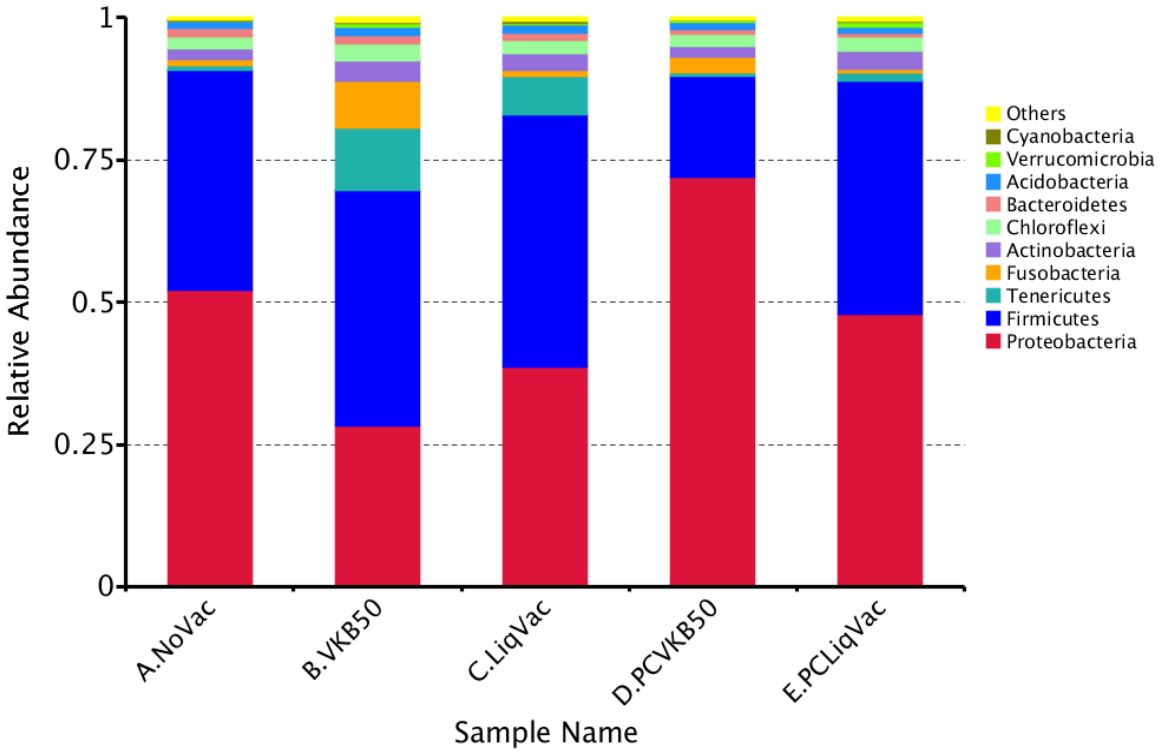

Figure 4. Phylum composition present in all treatments

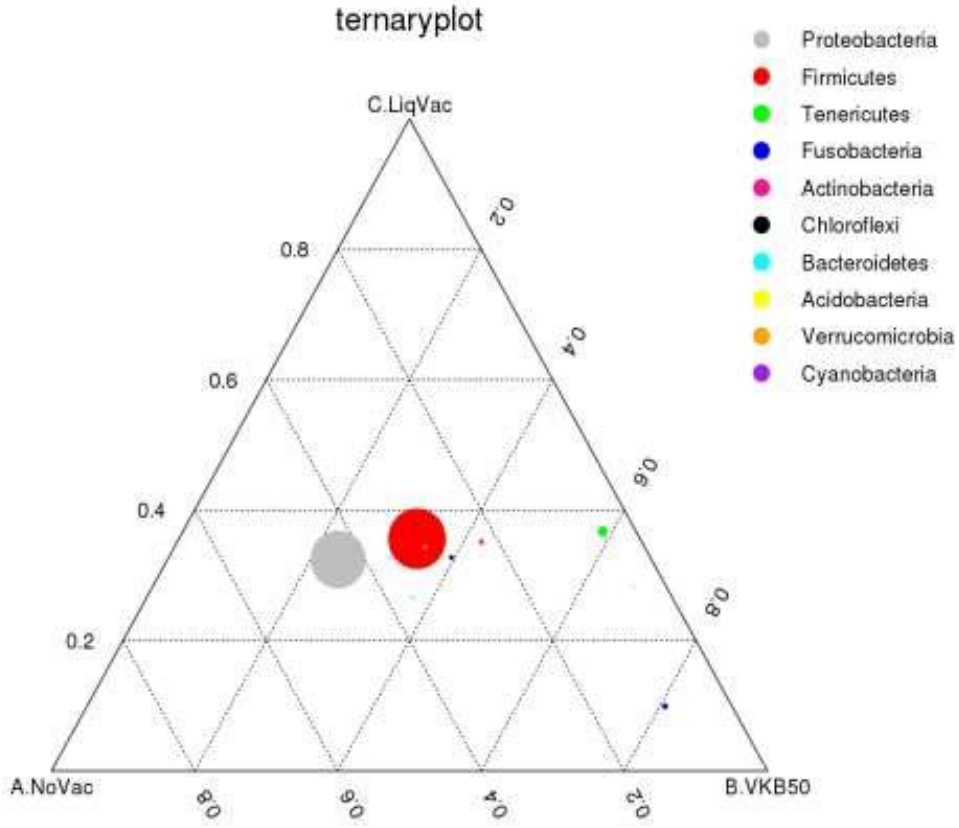

Figure 5. Phyla ternaryplot present in 3 treatment

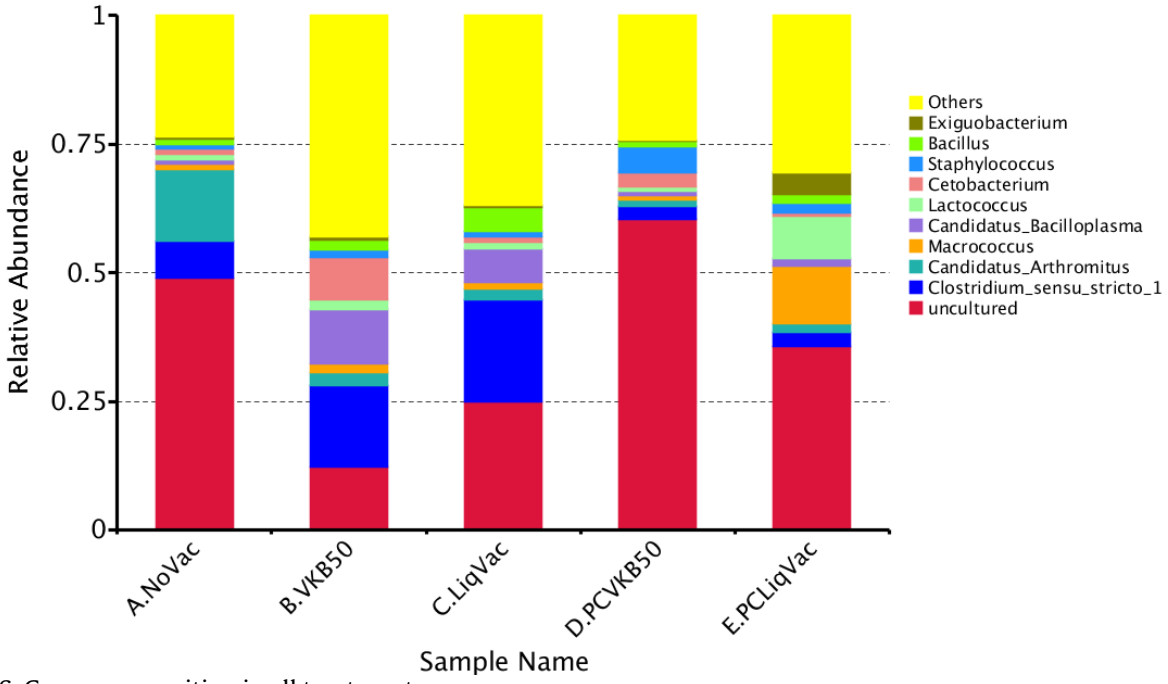

Figure 6. Genus composition in all treatments

and the genus Candidatus Bacilloplasma appeared in the fish group vaccinated with VKB50.

In Figure 7, some several important bacterial genera and species appear after being vaccinated, using either the liquid vaccine or the dry vaccine. The bacterial species in the liquid vaccine (LiqVac) treatment were *Bacillus clausii* and *Bacillus gibsonii*. In VKB50 treatment or dry vaccine, *Clostridium* clusters appeared, namely *Clostridium butyricum*, and others were genus *Cetobacterium* and *Candida bacilloplasma*.

4. Discussion

According to Llewellyn *et al.* (2014), the fish digestive tract mainly contains obligate anaerobic, facultative anaerobic and aerobic bacteria. Moreover, Ringo (1999) added that intestinal microbes are categorized into two groups, the allochthonous microbiome, which passes through the lumen via feed. The other is autochthonous microbiome, which is potentially resident and closely related with host tissue. Based on NGS data of intestinal microbes in freshwater fishes, includes the group of *Actinobacteria, Bacilli, Bacteroidetes, Clostridia, Firmicutes, Fusobacteria, Proteobacteria*, and *Verrucomicrobia*. From the 8 phyla, 5 phyla are dominant, namely *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes,* and *Fusobacterium* (Ringø *et al.* 2006; Desai *et al.* 2012; Li *et al.* 2013; Carda–Dieguez *et al.* 2014; Ingerslev *et al.* 2014a, 2014b), although the composition varies between different microbial species due to environmental conditions of the digestive tract and the type of feed consumed. Information on microbial diversity is expected to identify unique genera as adaptive microbial germplasm and to optimize the role of these microbes later in fish health management strategies to increase cultivation productivity. The gut microbiome has a significant role in digesting complex feed substances and maintaining the fish's health status. Changes in the host's dietary habits and health conditions lead to rapid changes in the taxonomic composition of the fish gut microbiome (Xia *et al.* 2014).

Chitosan was used as a coating agent for the *A. hydrophila* vaccine in this research. Hyean–Woo Lee *et al.* (2003) said that chitosan contains oligosaccharides that provoke the growth of several gut microbiome and can be prebiotic. Phylum *Proteobacteria* and *Firmicutes*, according to Wang *et al.* (2018), is a microbiome that can be in the digestive tract of healthy fish, namely, 44.33% and 9.11%, respectively, and the digestive tract of unhealthy fish, namely 70.46% and 7.55%. Asian Redtail Catfish (*Hemibagrus nemurus*) used in this research is a local fish widely cultivated in Indonesia. It has the same microbial composition in general as the digestive tract microbes of freshwater

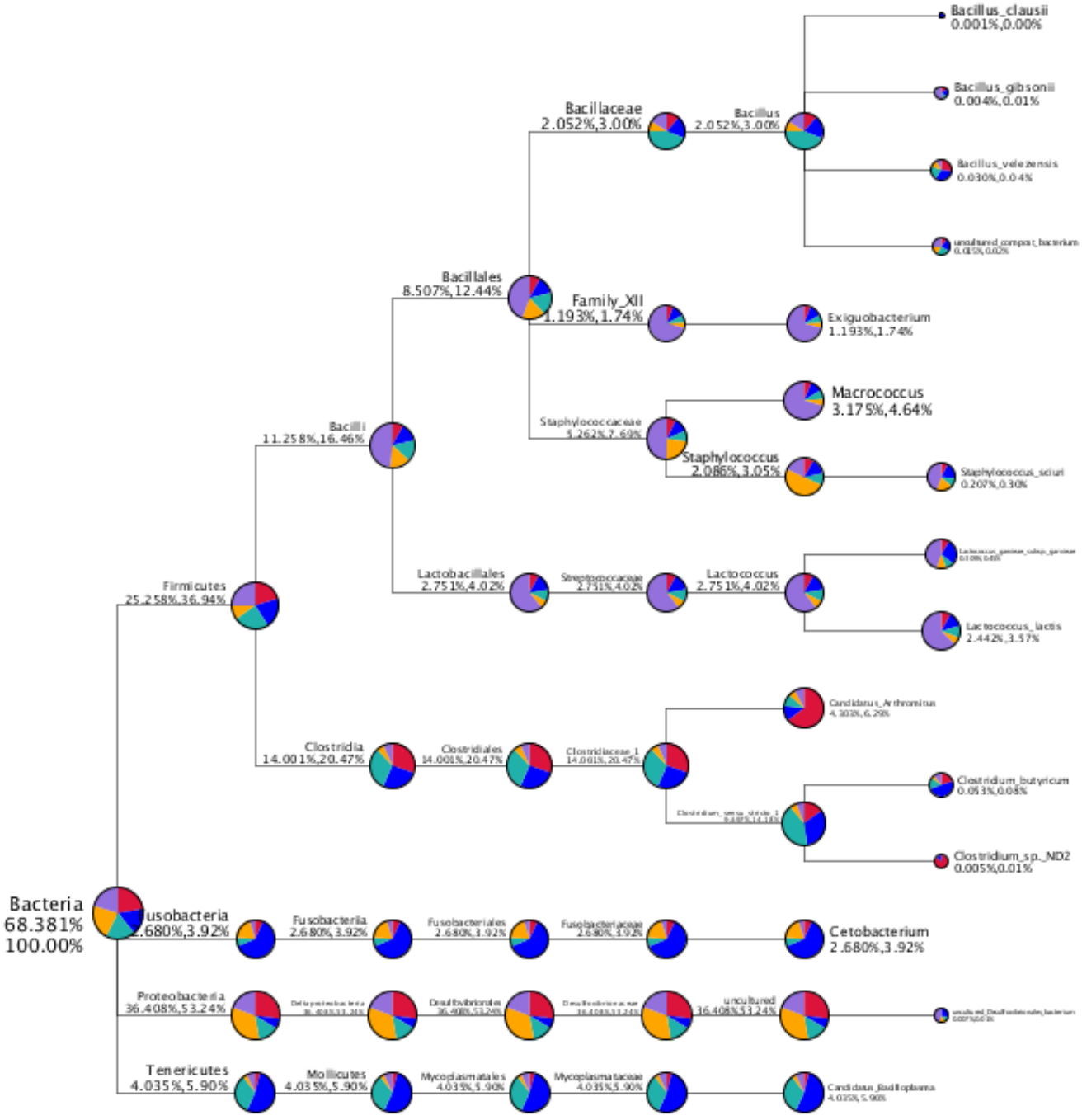

Figure 7. Taxtree of all treatments, several types identified to species

fish. Baung lives in public waters such as rivers, swamps, lakes, and reservoirs and is an omnivore fish. However, baung are also classified as carnivorous fish because they predominantly eat small fish, small shrimp, mussels, and insects. Furthermore, Wu *et al.* (2004) stated that a healthy microbiome increases the immune response to vaccination; for example, the successful implementation of the cholera vaccine is characterized by the presence of dominant Gram–negative bacteria in the gastrointestinal tract. So, the gut microbiome composition can be used to determine the effect of vaccination on fish health status and to obtain candidate probiotic bacteria for fish digestive tract health.

In all treatments from the results of this study, both control and treatment, two significant phyla were identified, namely *Proteobacteria* and *Firmicutes*, while the other phyla that appeared in the heat map

were the *Actinobacteria, Bacteriodetes, Cyanobacteria, Fusobacteria, Tenericutes*, and *Verrucomicrobia* groups. Meanwhile, Ringø *et al.* (2006); Desai *et al.* (2012); Li *et al.* (2013); Carda–Dieguez *et al.* (2014); Ingerslev *et al.* (2014a, 2014b) were found the same phyla except for Cyanobacteria and Tenecurites. The types of bacteria between control and treatment were the same, but the number of bacteria was different. In vaccination treatment, both phyla and genera increased the number of bacteria than control. Freeze–dried vaccines tend to have more bacteria than liquid vaccines. It is because the freeze–dried vaccine uses chitosan as an antigen coating of *A. hydrophila*. According to Yang *et al.* (2012), chitosan improves intestinal function as a pathogenic barrier, increases the population of bacteria that can improve the health status of hosts such as *Lactobacillus* sp., and reduce the number of pathogenic bacteria.

This research showed that 11 genera appeared in all treatments besides the "uncultured" and "others" genus. There were nine genera, namely *Exiguobacterium, Bacillus, Staphylococcus, Cetobacterium, Lactococcus, Candidatus Bacilloplasma, Candidatus Arthromitus, Macrococcus,* and *Clostridium sensu stricto*. It is different from the results obtained by Austin (2006); and Gomez and Balcazar (2008). They found *Aeromonas, Acinetobacter, Bacteroides* type A, *Bacteroides* type B, *Clostridium, Enterobacteriaceae, Fusarium, Micrococcus, Pseudomonas*, and *Plesiomonas*. From this research, there are three genera whose numbers dominate the vaccinated fish, namely *Cetobacterium, Candidatus Bacilloplasma,* and *Clostridium sensu stricto*. Tsuchiya *et al.* (2008) and Van Kessel *et al.* (2011) are found the genus *Cetobacterium*, including *Cetobacterium somerae* in freshwater fish such as *Oreochromis niloticus* and *Cyprinus carpio*. Cetobacterium can produce vitamin B12 (Sugita *et al.* 1991) and become a barrier for the growth of other bacterial strains (Sugita *et al.* 1996). The existence and abundance of freshwater fish are potential in developing probiotics in the future (Larsen *et al.* 2014).

Apart from *Cetobacterium, Candidatus Bacilloplasma* is also dominant in the gastrointestinal tract of baung fish. According to Infante–Villamil *et al.* (2016), *Candidatus Bacilloplasma* is a probiotic candidate that affects survival, growth, and water quality in pond productivity. *Candidatus Bacilloplasma* is a new type of bacteria from the *Mollicutes* group, which is rod–shaped and forms colonies on the intestine's surface in mutualism symbiosis with other bacteria from the same group. Colonization of *Mollicutes*, including *Candidatus Bacilloplasma*, when the digestive tract is actively working. This colonization suggests a close relationship between bacterial colonization and digestive tract function (Kostanjsek *et al.* 2007).

Bacillus is the dominant bacteria in the liquid vaccine group, and according to Mingmongkolchai and Panbangred 2018, *Bacillus* is often used as a probiotic. *Bacillus* is a member of the *Firmicutes* family and is often used as digestive probiotics in aquaculture (Tyagi *et al.* 2015; Ringo *et al.* 2018). Probiotic bacteria may be competitors to pathogens in utilizing nutrients. They can improve the fish's immune system and may protect fish from infection through enhanced immunity. The use of probiotics as disease prevention and control is environmentally friendly and can reduce antibiotics. Two species, bacteria *Bacillus clausii*, and *Bacillus gibsonii* were found in the vaccinated group. *Bacillus clausii* and *Bacillus gibsonii* are Gram positive, rod–shaped, non–pathogenic, spore–forming, aerobic, acid resistant and very alkaline conditions and antibiotic therapy conditions. Moreover, *B. gibsonii* grows in a pH range of 7–12 and a temperature range of 4–40°C and can utilize sugar beet pulp as a carbon source and stimulates pectinase to produce extracellular alkaline pectinases (Duc *et al.* 2004).

The digestive tract microbiome of fish can produce various enzymes such as amylase, cellulase, lipase, protease, chitinase, and phytase, which have functions in digestion, metabolism, absorption of essential nutrients cholesterol (Rawls *et al.* 2004; Ray *et al.* 2012).

The other dominant genus in the freeze–dried vaccine treatment group was *Clostridium sensu stricto* and *Clostridium butyricum. Clostridium sensu stricto* can utilize polysaccharides, such as cellulose, xylan, and hemicellulose, as saccharolytic, fermentative, and proteolytic (Li *et al.* 2015). *Clostridium butyricum* can lower cholesterol levels, prevent cancer and treat infections due to *Clostridium difficile* (Guo *et al.* 2020). Both *Clostridium sensu stricto* and *Clostridium butyricum* are members of Clostridium XIVa and IV groups which account for 10 to 40% of the total beneficial gut microbiome.

Clostridium is a commensal and chemoorganotropic bacteria that fermenting various feed nutrients to produce acetic acid, propionic acid, butyric acid, acetone, and butanol (Guo *et al.*

2020). Moreover, Semova *et al.* (2012) stated that *Clostridium sensu stricto* increases the absorption of fatty acids in the intestinal epithelium, causing an increase in lipid droplets in enterocyte cells and the accumulation of dietary fatty acids in the extraintestinal tissue.

In this research, vaccination increases the number and diversity of gut microbiome that modulates the immune response of Asian redtail catfish, *Hemibagrus nemurus*. The characteristic of vaccines, both liquid or freeze–dried, can influence the diversity of gut microbiome and increase immunity by stimulating and exploring the microbiome's ability to support the development of both innate and adaptive immune systems.

As shown in Figure 5, the Genus composition is dominated by red and yellow colors, which refer to the "uncultured" and "others" genus. It indicates that there are new isolates that have not been described or given a valid name. So, the genus cannot be included as a species name in the taxonomic framework corresponding to the microbe. Many bacteria cannot be classified into genus level and even family level. Therefore, these microbes are classified as "others" or "not cultivated" in a particular phylum, class, order, or family. For example, Kim *et al.* (2011) classify rumen bacteria in the 16S rRNA gene sequence at the genus level but are constrained by many rumen bacterial sequences that cannot yet be classified. Sequences classified at the genus level show low sequence similarity, which means that their taxonomic resolution needs to be increased (Collins *et al.* 1994; Yutin and Galperin 2013). The genus level is often used for taxonomic determination of next–generation sequencing data, especially from samples with short read lengths (200 to 400 bp), making a resolution at the species level unreliable because it depends on the 16S rRNA gene region used (Kim *et al.* 2011).

According to Sullam *et al.* (2012), Ringø *et al.* (2016), Dehler *et al.* (2017) digestive tract microbiome of fish is influenced by host factors such as sex, body weight, and age. Environmental factors such as water quality, frequency of feeding, antibiotic therapy also play a role. In addition, Prakash *et al.* 2011 stated that microbial factors such as being able to penetrate cell walls and producing enzymes for cell metabolism processes as well as individual variations and day–to–day fluctuations (Sugita *et al.* 1987; Sugita *et al.* 1990; Ringø *et al.* 1995; Ringø 1999) also influence the microbiome composition of the digestive tract. According to Rawls *et al.* (2004), the gastrointestinal microbiome regulates gene expression, which plays a role in stimulating epithelial proliferation, enhancing nutrient metabolism, and regulating innate immune responses. Intestinal epithelial cell dysfunction, nutrient absorption, metabolism, and weak immune response are probably due to the low diversity and number of microbiomes. Cheesman *et al.* (2011) studied that the developing intestine of zebrafish can be stimulated by the presence of intestinal microbiome and activation of signals that induce the accumulation of b–catenin in the cytoplasm of cells. Furthermore, Cheesman *et al.* (2011) stated that the resident gut microbiome increases the proliferation and stability of b–catenin in intestinal epithelial cells.

Ciabattini *et al.* 2019 in Figure 8 explain that the interaction between the microbiome and host cells occurs on the surface of the intestinal epithelium, stimulating and releasing various immune factors that play a role in the homeostasis of the intestinal immune system. The epithelial surface of the intestine is covered with a layer of mucus produced by goblet cells (GC). Microbial related molecular patterns (MAMPs), expressed on the surface of bacteria, are recognized by pattern recognition receptors (PRR), which are described by intestinal epithelial cells (IEC). This process causes the blocking of bacteria through the production of antimicrobial peptides (AMPs). The factors released by IEC, namely retinoic acid (RA) and TGF–b, cause lamina propria to develop tolerogenic DC and differentiate T cells to become Tregs. B cells that differentiate into plasma (PC) cells produce IgA and are released into the mucus layer, causing bacteria to the host tissue. Flagellin signaling prompts macrophages to secrete IL–23, and ILC3 produces IL–22 to release RegIIIg, an antimicrobial peptide produced by IEC. ILC2 releases IL–13 for mucus control. Cytokines stimulate the differentiation of intestinal epithelial stem cells into goblet cells (GC), a source of mucin glycoproteins.

Intestinal lymphoid tissue (GALT) protects the GI tract from pathogenic infections and coordinates the immune system in the GI tract. The role of the gut microbiome in mediating various factors that regulate immunity is also essential in the process of growth and development, and maturation of GALT. Therefore, studies are needed to observe the emergence of beneficial gut microbiome due to vaccines that play a role in increasing fish immunity.

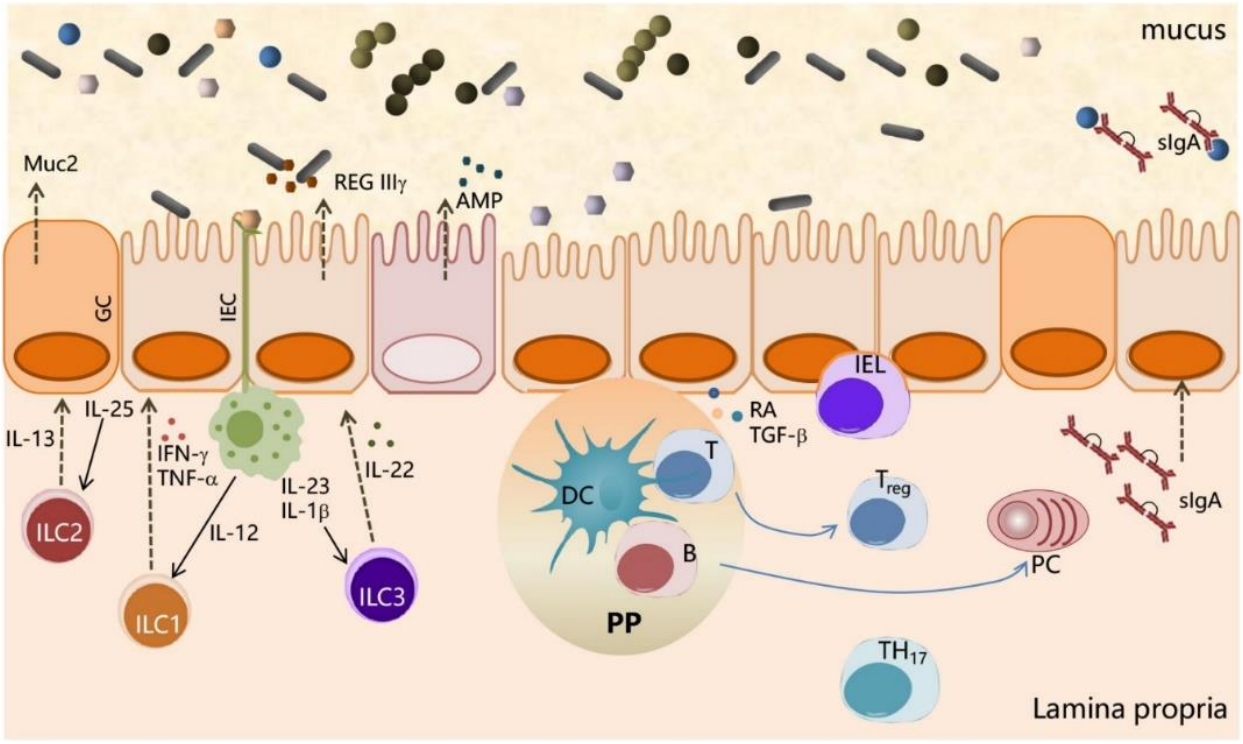

Figure 8. Interactions between the microbial community and the immune system at mucosal surfaces

The contribution of *Bacillus clausii* and *Bacillus gibsonii*, which are dominant in liquid vaccine application, also *Clostridium, Cetobacterium,* and *Candida Bacilloplasm* clusters in dry vaccines that play a role in fish health can be further investigated. The intestinal microbiome, which has a role in boosting immunity can be developed as candidates for the next generation of probiotics (NGPs) and is expected to produce future anti–microbials.

In conclusion, vaccine application increases the composition and diversity of the gut microbiome, which modulates the immune response of *Hemibagrus nemurus*. Good bacteria appear in both liquid and freeze–dried vaccine applications. Dried vaccines using chitosan as a coating antigen increase the number and diversity of gut microbiome better than the liquid vaccine.

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