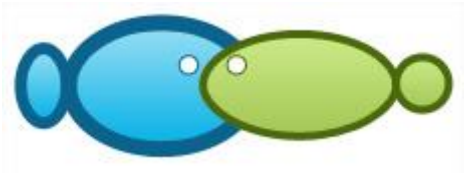

Bacteria associated with *Trichodina* sp. infection of barramundi, *Lates calcarifer* in a fish farm in South Sulawesi, Indonesia

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Abstract. Bacteria is one of the disease agents that frequently attack aquaculture commodities including barramundi (*Lates calcarifer*) in aquaculture facilities. Diseases caused by bacterial infection or co-infection with other parasites are known to cause severe health problems in aquaculture industry. Identification of pathogens plays an important role for the prevention of transmission and for the fish disease therapeutic accuracy. The study aimed to identify bacteria in cultured *L. calcarifer* and to analyze their relationship with *Trichodina* infection rate and fish size. The samples were collected from the Takalar fish farm (South Sulawesi, Indonesia) and transported using sterile plastic containers filled with seawater and having an aeration systems, then analyzed at the Fish Parasites and Diseases Laboratory, Hasanuddin University, Makassar, Indonesia. Bacteria were isolated from gills and mucus based on the criteria of the parasitic infection rate in the fish specimens. The marine agar media used to grow bacteria was incubated at 32^{o}C for 24-48 hours. The bacterial colonies were purified on blood agar media, then a morphological characterization and a Gram staining were performed. The biochemical characterization of the selected isolate was performed using the rapid identification of microorganisms by a Vitek-2 compact system. This study shows that all of the 8 species of identified bacteria from the mucus and gills of infected fish belong to the *Bacillus* genus. The variance of the parasites' infection rate shows an impact on the number of bacteria ($P<0.05$). Our finding also demonstrated a tendency to increase in bacteria and parasite numbers with the increase of the host size ($P<0.05$). The results show the relation of the bacterial infection with the ciliated *Trichodina* in *L. calcarifer* aquaculture and confirmed that an increase of the parasite infection rate caused the increase of bacterial density in *L. calcarifer*.
Key Words: fish disease, marine aquaculture, parasite, Vitek-2.

Introduction. Barramundi (*Lates calcarifer)* is one of the most important economic commodities in the marine aquaculture sector (Toranzo et al 2005). *L. calcarifer* is widely cultured in Indonesia because it has several advantages, such as high market value, wide physiological tolerance range, high fecundity and relatively fast growth (Hidayat et al 2014). However, *L. calcarifer* culture is also susceptible to be infected, which cause diseases, interfering with the fish farming sustainability.

There are many factors that may contribute to the fish diseases, including bad environmental conditions, poor feed quality or inadequate broodstock. Besides the use of inappropriate cultivation techniques, contamination from cultivation tools and workers can also cause a rapid transmission of diseases (Chatterjee & Haldar 2012). It has been reported that *L. calcarifer* has experienced a significant decrease in the last decade due to bacterial outbreaks (Toranzo et al 2005).

Parasitic infection is a frequent disease in fish farming environments. Ciliated parasites such as *Trichodina* are often found to infect cultured fish in the world, in both freshwater and sea water environments. Generally, *Trichodina* is an ectoparasite that attacks gills or the surface body of fish (Kotob et al 2016). *Trichodina* sp. infection is considered highly pathogenic because it may cause high significant mortality in cultured fish (Xu et al 2015). In Indonesia, *Trichodina* sp. infection of *L. calcarifer* has been widely reported (Rueckert et al 2008).

In an aquatic environment, bacteria are pathogenic agents that often co-occur with other pathogens. Among them, the genera of *Bacilli* and *Coccus* bacteria, such as *Aeromonas* sp. and *Streptococcus* sp. are often found to affect aquaculture fish. The bacterial disease is known to cause several problems, often lethal, in the affected fish such as body surface damage, loss of appetite, bleeding, and swelling in certain organs such as gills, kidneys and spleen. It can increase the mortality of fish and decrease the production of the aquaculture industry (Ashari et al 2014). Other potentially disease-causing bacterial agents from the *Bacillus* genus include: *Aeromonas* sp., *Pseudomonas fluorescens*, and *Streptococcus iniae*, that are responsible for motile aeromonad septicemia (AMS) and bacterial hemorrhagic septicemia (Foysal et al 2011).

Spore-forming *Bacillus* spp., which are Gram-positive, aerobic or facultative anaerobic bacteria, have advantages because the sporulation stage of their life cycles is stable to heat, stomach acid and other harsh environments (Cui et al 2019). Yifang et al (2020) stated that more than 60% of the bacteria isolates (39 isolates of the 65 studied, of which 36 belonging to the *Bacillus* genera) showed a hemolytic activity, which may be attributed to the bacterial virulence factors, and produced enterotoxins and various cytotoxic surfactin-like toxins. Then, it has been described that isolates from the *Bacillus* genus are known for their ability to produce mainly four toxins, including three enterotoxins (Hbl, Nhe, and CytK1) and one peptide toxin (cereulide) (Bottonee 2010).

Bacterial infections are also reported to be very likely to have interactions with other pathogens (parasites and virus) in fish breeding ponds, and these interactions even increase the presence of various deadly diseases in cultured organisms (Kotob et al 2016). Bowden et al (2007) stated that *Trichodinid ciliates* parasite caused epithelium damage to fish gills and caused secondary bacterial infection.

Microorganisms are a part of the ecological community, therefore they have a complex relationship between them and also with other microorganisms. The metabolites of one microbe facilitate the feeding of the others, resulting in mutualism. Under extreme conditions, other organisms may be more likely to acquire favorable factors, leading to rapid growth and reproduction, finally becoming the dominant species in the environments, while the progressive microorganisms degrading is inhibited (Mekuto et al 2018). Conversely, the interaction of parasite infection with the presence of bacteria in the fish host and their possible impact on the disease outbreaks are poorly understood (Soto et al 2012; Colquhoun et al 2011).

To date, information on the pathogenicity relationship between bacterial infection and the ciliated parasite *Trichodina*, especially in the *L. calcarifer* culture in Indonesia is limited. We hypothesized that infection of *Trichodina* might enhance the susceptibility of the *L. calcarifer* to opportunistic bacteria. The present study aimed to identify bacteria in cultured *L. calcarifer* and to analyze the potential interactions between the parasites and bacteria present in the *L. calcarifer* from Takalar fish farm, Indonesia.

Material and Method

Location and sample collection. Fish samples were collected from the Takalar fish farm, located around 40 km from Makassar City, Indonesia. The fish samples were transported to the Parasite and Fish Disease Laboratory, Department of Fisheries Science at Hasanuddin University of Makassar, Indonesia. Sterile plastic containers filled with aerated seawater were used during the transportation, keeping the fish alive until bacterial and parasitological examinations were performed.

Bacterial isolation. Isolation of bacteria was conducted using aseptical marine agar media in laminar air flow. Bacteria isolated from the gills and mucus were diluted (three multilevel dilution) using 0.9% NaCl physiological solution. Samples were categorized into three categories based on the level of parasites infection of the fish: 0-5 parasites fish^{-1} (light infection), 12-15 parasites fish^{-1} (moderate infection) and >85 parasites fish^{-1} (heavy infection). Bacterial isolation was carried using serial dilutions. 100 µL were taken from the diluted tube using a micropipette, then inoculated onto a Petri dish containing marine agar and spread it evenly until blended, then incubated at 32^{o}C for 24–48 hours.

Different colonies of bacteria grown on agar plates were selected based on the dominance of their morphological characters and purified. Bacterial purification was carried out on a blood agar media (BAM) plate using the streak plate method for further identification. Identification of bacteria was carried out initially through Gram staining and then performed biochemical tests using Rapid Identification of Microorganism by Vitek-2.

Morphological characterization. Morphologically, the characteristics of bacterial colony were observed based on color, shape, transparency, elevation and margin shape according to Cappuccino & Sherman (1987). In addition, Gram staining was also performed for morphological cell investigation of bacteria.

Biochemical characterization. Biochemical characterization was carried out using a Rapid Identification of Microorganisms by Vitek-2 Compact. This analysis was done based on the results of the Gram staining, which used identification cards for Gram-negative (GN) and Gram-positive (GP) bacteria.

The brief protocol for bacterial test using the Vitek-2 Compact system comprised the following steps: suspension of bacteria from pure isolates was taken using sterile cotton swabs and re-suspended into disposable tubes containing Sodium Chloride (NaCl) to commensurate with the standard turbidity of 0.48-0.56 on the McFarland scale (measured using DensiCHECK). Then, each 64-Well Vitek-2 disposable target slide (identification card) was installed into each disposable tube and moved to the cassette (Figure 1) and the cassette was inserted into a Vitek-2 compact instrument. The results of identification were known in a period of less than 24 hours (10-12 hours). Calibration and quality control for every group of bacteria was done using ID cards of Gram-negative and Gram-positive bacteria based on Gram staining result.

Figure 1. Identification card and disposable tube of Vitek-2 Compact along with the cassette (original).

Data analysis. Some parameters were analyzed statistically, using the Analysis of variance (ANOVA). The parameters were: the number of bacteria at different levels of parasite infection, the number of bacteria for different fish sizes and the mean intensity of the parasites in relation to the fish size. However, a non-parametric one-way ANOVA, the Kruskal-Wallis test by ranks, with a 95% confidence interval and significance level of 0.05) is performed when the normality and homogeneity of the data are not fulfilled. The statistical analysis was performed using the Statistical Package for the Social Science (SPSS) version 25. The density of bacteria was calculated using the formula (Fardiaz 1993):

$$\text{CFU/mL} = \left(\text{Bacteria number } \text{x} \frac{1}{\text{dilution factor}}\right) \text{x } 10$$

Results

There were 8 species of bacteria found from the gills and mucus of *L. calcarifer*. The morphology of the colonies of all bacterial isolates is shown in Figure 2 and Table 1.

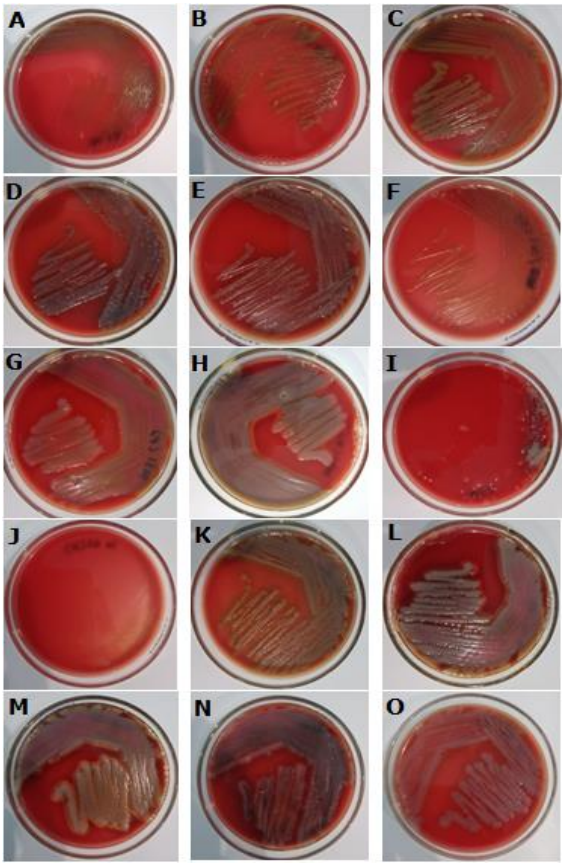

Figure 2. Pure isolate of bacteria from gills and mucus on BAM. A) Gills 1.2W; B) Gills 2.1Y; C) Gills 2.2Y; D) Gills 2.2 W; E) Gills 3.2 Y; F) Gills 3.2W; G) Gills 4.1Y; H) Gills 4.1W; I) Mucus 1Y; J) Mucus 2.1Y; K) Mucus 3.1Y; L) Mucus 3.1W; M) Mucus 4.1Y; N) Mucus 4.1W; O) Mucus 5.2W.

Table 1

Morphological character of bacterial colonies

No.	*Isolate code*	*Color*	*Form*	*Margin*	*Elevation*	*Surface texture*	*Size*
1	Gills (1.2 W)	White	Circular	Entire	Convex	Smooth	Large
2	Gills (2.1 Y)	Yellow	Circular	Entire	Flat	Smooth	Large
3	Gills (2.2 W)	White	Circular	Serrate	Raised	Rough	Large
4	Gills (2.2 Y)	Yellow	Circular	Entire	Flat	Smooth	Large
5	Gills (3.2 W)	White	Circular	Undulate	Flat	Smooth	Large
6	Gills (3.2 Y)	Yellow	Circular	Irregular	Flat	Smooth	Large
7	Gills (4.1 W)	White	Circular	Serrate	Flat	Smooth	Large
8	Gills (4.1 Y)	Yellow	Circular	Entire	Flat	Smooth	Small
9	Mucus (1 Y)	Yellow	Circular	Serrate	Flat	Smooth	Small
10	Mucus (2.1 Y)	Yellow	Circular	Entire	Flat	Smooth	Small
11	Mucus (3.1 W)	White	Circular	Entire	Flat	Smooth	Small
12	Mucus (3.1 Y)	Yellow	Circular	Entire	Flat	Smooth	Small
13	Mucus (4.1 W)	White	Circular	Entire	Raised	Rough	Large
14	Mucus (4.1 Y)	Yellow	Circular	Entire	Flat	Smooth	Small
15	Mucus (5.2 W)	White	Circular	Entire	Flat	Rough	Large

The selection of isolates for further identification was based on the dominant characters of the colony: pigmentation and size. Then, as representative of size, large and small colonies were selected in each plates.

Cell characterization of bacteria was carried out through Gram staining and it was found that all cells of bacteria had a basil form, except for 1 which was in the form of coco-basil. Of the 15 isolates stained, 7 isolates were classified as Gram-negative and 6 isolates were Gram-positive while the other 2 isolates were mixed, with 2 groups of Gram negative and positive bacteria, as shown in the isolates 3.2 Y and 4.1 Y from gills (Figure 3). In the present study, 8 species of bacteria that live in the gills and mucus of *L. calcarifer* have been identified using the Vitek-2 compact instrument with biochemical characteristics, as shown in Table 2.

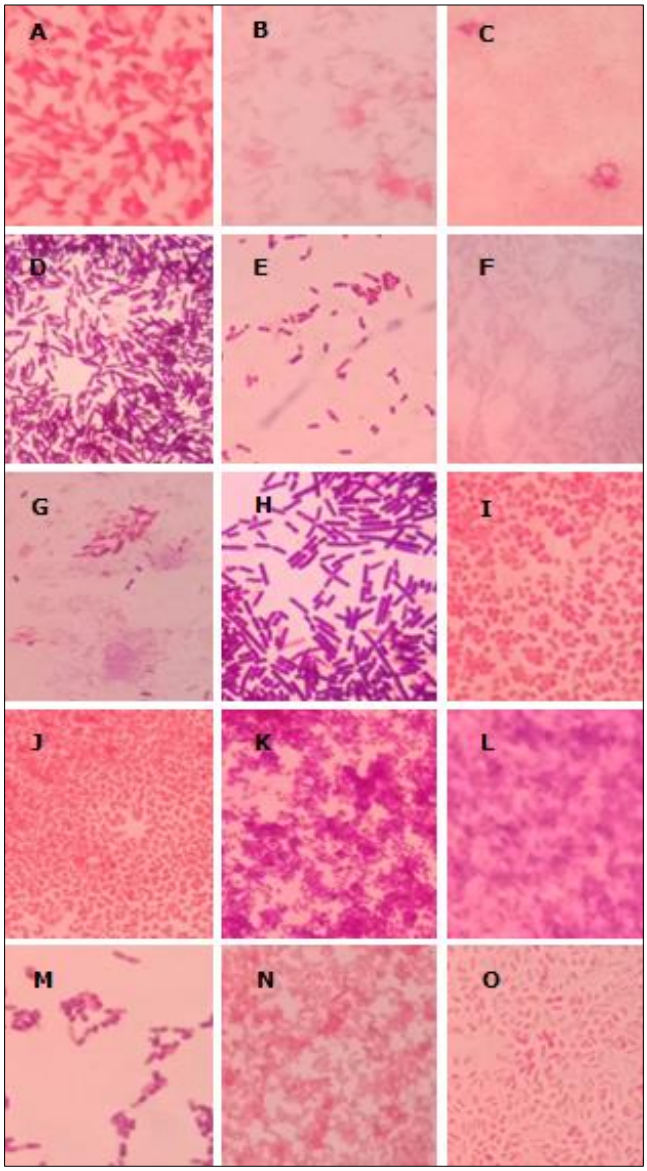

Figure 3. Gram staining of bacteria from gills and mucus. A) Gills 1.2W; B) Gills 2.1Y; C) Gills 2.2Y; D) Gills 2.2 W; E) Gills 3.2 Y; F) Gills 3.2W; G) Gills 4.1Y; H) Gills 4.1W; I) Mucus 1Y; J) Mucus 2.1Y; K) Mucus 3.1Y; L) Mucus 3.1W; M) Mucus 4.1Y; N) Mucus 4.1W; O) Mucus 5.2W.

After the Gram staining was carried out, it was continued with a biochemical test using the Vitek-2 compact instrument. The results of the biochemical test are shown in Table 2.

Table 2

Biochemical test results using *Bacillus* GN and GP identity card by Vitek-2 compact system

Biochemical test		*Isolate code of GN bacteria*						*Biochemical test*		*Isolate code of GP bacteria*			
		G1.2W	*G2.1Y*	*M1Y*	*M2.1Y*	*M4.1W*	*M5.2W*			*G2.2W*	*G3.2W*	*G3.2Y*	*G4.1W*
APPA	IARL	-/-	-/-	-/-	-/-	-/-	-/-	BXUL	LeuA	-/-	-/+	-/+	-/+
H2S	Dglu	-/+	-/+	+/+	-/+	-/+	-/+	BGAL	AlaA	-/-	-/+	-/+	-/-
BGLU	dMNE	-/+	-/+	+/+	+/-	-/+	-/+	APPA	GLYG	-/+	-/-	+/-	-/+
ProA	TyrA	+/-	+/+	-/-	-/-	+/+	-/-	ELLM	MTE	-/+	+/-	+/-	+/+
SAC	CIT	+/-	-/+	+/-	+/-	-/+	-/-	dMNE	PLE	-/-	-/-	-/-	-/-
ILATk	NAGA	-/-	+/-	-/-	-/-	+/-	-/-	BMAN	AGLU	-/(+)	-/-	-/-	-/+
GlyA	IHISa	-/-	-/+	-/-	-/-	-/+	-/-	INU	PSCNa	-/-	-/-	-/-	-/-
O129R	ELLM	-/-	+/-	+/+	-/(-)	+/-	-/-	OLD	POLYB_R	-/+	-/-	-/-	-/+
ADO	dCEL	-/-	-/-	-/+	-/-	-/-	-/-	LysA	PheA	-/+	-/+	+/+	-/+
BNAG	GGT	-/-	-/+	+/-	+/-	-/+	+/-	PyrA	TyrA	+/-	+/+	(-)/+	+/+
dMAL	BXYL	+/-	-/-	+/-	-/-	-/-	+/-	CDEX	INO	-/-	-/-	-/-	-/-
LIP	URE	-/-	-/-	-/-	-/-	-/-	(-)/-	MdX	GlyA	-/-	-/-	-/+	-/-
dTAG	MNT	-/-	-/+	-/-	-/-	-/+	-/-	dMLZ	IRHA	/-	-/-	-/-	-/-
AGLU	AGAL	-/-	-/-	+/-	+/-	-/-	-/-	PHC	dTAG	-/-	-/-	+/-	-/-
ODC	CMT	-/+	-/+	-/+	-/+	-/+	-/+	dGLU	NaCl 6.5%	+/+	-/-	-/-	+/+
GGAA	ILATa	-/-	+/+	-/-	-/-	-/+	-/-	ESC	ProA	+/-	-/-	-/-	+/-
PyrA	BGAL	-/-	-/-	-/-	-/-	-/-	+/-	AspA	BNAG	-/+	+/-	+/+	-/+
AGLTp	OFF	-/-	+/-	-/-	-/-	-/-	-/+	AGAL	MdG	-/-	-/-	+/-	-/-
dMAN	BAlap	-/-	-/+	+/-	-/-	-/-	-/-	dGAL	dMAN	-/-	-/-	+/-	-/-
PLE	dSOR	-/-	-/-	-/-	-/-	-/-	-/-	AMAN	BGLU	-/-	-/-	+/-	-/-
dTRE	5KG	+/-	+/-	+/-	-/-	-/-	+/-	NAG	dTRE	+/+	-/-	-/-	+/+
SUCT	PHOS	-/-	+/-	-/-	-/-	+/-	-/+	PVATE	KAN	+/-	-/-	+/-	+/+
LDC	BGUR	-/-	-/-	-/-	-/-	-/-	+/-	dRIB		+	-	-	+
IMLTa		-	+	-	-	+/-	-	TTZ		+	-	-	+

Bacterial identification according to the analysis of Vitek-2 compact system. Based on the results of Gram staining and biochemical characterization using the compact Vitek-2 system, 8 species of bacteria were identified from mucus and gills of *L. calcarifer* (Table 3).

The most commonly found bacteria in this study were from the *Bacillus* genus. The species of bacteria found on the *L. calcarifer* gills were: *Spingomonas paucimobilis* (96% probability), *Pseudomonas aeroginosa* (92% probability), *Bacillus* sp. (93% probability), *Brevibacillus choshinensis* (98% probability), *Bacillus* sp. (97% probability) and *Lysinibacillus fusiformis* (89% probability), whereas the species of bacteria found from the mucus samples were: *Aeromonas caviae* (95% probability), *Sphingomonas paucimobilis* (96% probability), *Pseudomonas putida* (99% probability), *Photobacterium damselae* (95% probability). One isolate was unidentified. The probability percentage represents the accuracy level of the species identification.

Table 3

Bacterial identification results according to Vitek-2 compact system

Isolate code	*Gram staining*	*Species of bacteria*
Gills (1.2 W)	Basil Gram negative	*Spingomonas paucimobilis*
Gills (2.1 Y)	Basil Gram negative	*Pseudomonas aeroginusa*
Gills (2.2 W)	Basil Gram positive	*Bacillus* sp.
Gils (2.2 Y)	Basil Gram positive	Unidentified bacteria*
Gills (3.2 W)	Basil Gram positive	*Brevibacillus chosinensis*
Gills (3.2 Y)	Basil Gram positive	*Licinubacillus fusiformis*
Gills (4.1 W)	Basil Gram positive	*Bacillus* sp.
Gills (4.1 Y)	Mixed	Unidentified bacteria*
Mucus (1 Y)	Coco-basil Gram negative	*Aeromonas caviae*
Mucus (2.1 Y)	Basil Gram negative	*Spingomonas paucimobilis*
Mucus (3.1 W)	Basil Gram positive	Unidentified bacteria*
Mucus (3.1 Y)	Basil Gram negative	Unidentified bacteria*
Mucus (4.1 W)	Basil Gram negative	*Pseudomonas putida*
Mucus (4.1 Y)	Mixed	Unidentified bacteria*
Mucus (5.2 W)	Basil Gram negative	*Photobacterium damselae*

* Of the 15 isolates analyzed, 5 isolates were unidentified. Among them, 2 isolates from gills (2.2 Y, 4.1 Y) and 3 isolates from mucus (3.1 W, 3.1 Y and 4.1 Y), respectively.

The relationship of bacterial density with parasitic infection rates and host size. Bacterial count analysis on the sample of gills and mucus were done based on the fish size and infection rate of *Trichodina* (Figure 4). The results of bacterial density in CFU mL^{-1} are shown in Table 4.

Table 4

Bacterial number related to parasites number and size of infected fish by *Trichodina*

Sample code	*Fish length (cm)/∑ parasite*	*Dilution factor*	*Sample*		*Total (CFU mL^{-1})*	
			Gills	*Mucus*	*Gills*	*Mucus*
1.1	9.5/82	10^{-2}	235	241	$2.35x10^5$	$2.41x10^5$
1.2	9.5/82	10^{-3}	93	95	$9.3x10^5$	$9.5x10^5$
2.1	8.5/15	10^{-2}	243	232	$2.43x10^5$	$2.32x10^5$
2.2	8.5/15	10^{-3}	20	16	-	-
3.1	7.1/12	10^{-2}	110	229	$1.1x10^5$	$2.29x10^5$
3.3	7.1/12	10^{-3}	24	12	-	-
4.1	6.5/5	10^{-2}	201	244	$2x10^5$	$2.44x10^5$
4.2	6.5/5	10^{-3}	46	18	$4.6x10^5$	-
5.1	2.8/0	10^{-2}	13	20	-	-
5.2	2.8/0	10^{-3}	6	18	-	-

(-)Bacteria number is not eligible for SPC (30-300 colonies).

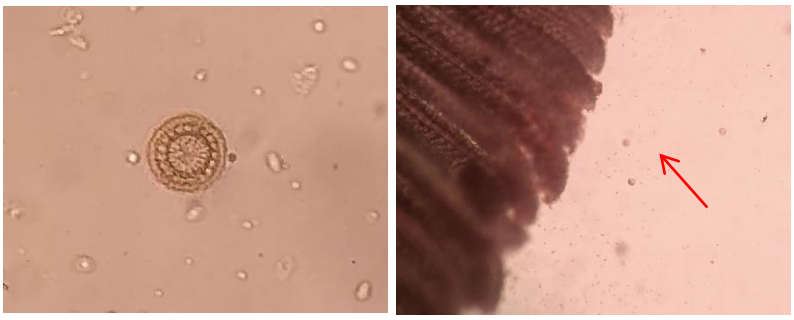

Figure 4. *Trichodina* on gills of *Lates calcarifer* from Takalar fish farm, Indonesia.

The non-parametric statistical test was used in this study to determine the differences between the number of bacteria at several parasitic infection rates. It was also applied to find out each difference for the number of bacteria based on different fish sizes and the mean intensity of parasite based on fish length interval. The results of the statistical analysis are represented in Figures 5, 6, and 7.

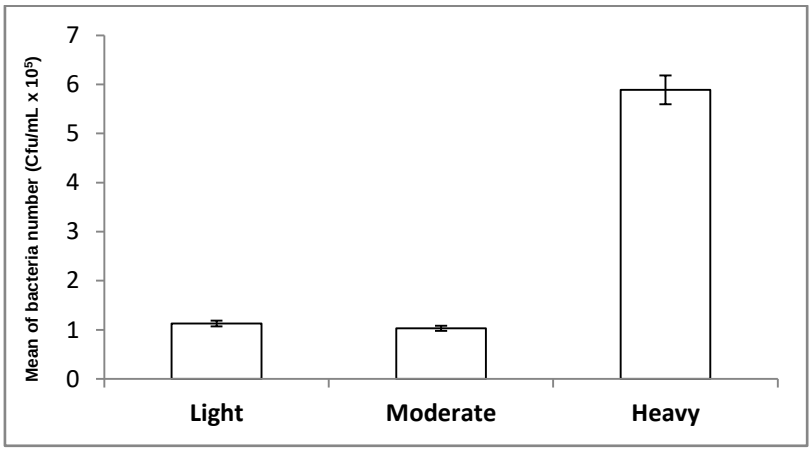

Figure 5. Number of bacteria based on parasites infection rates (P value:0.039). Classes=Light: 0-5 parasites/fish, Moderate 12-15 parasites/fish, Heavy:>80 parasites/fish. (No parasites except in categories).

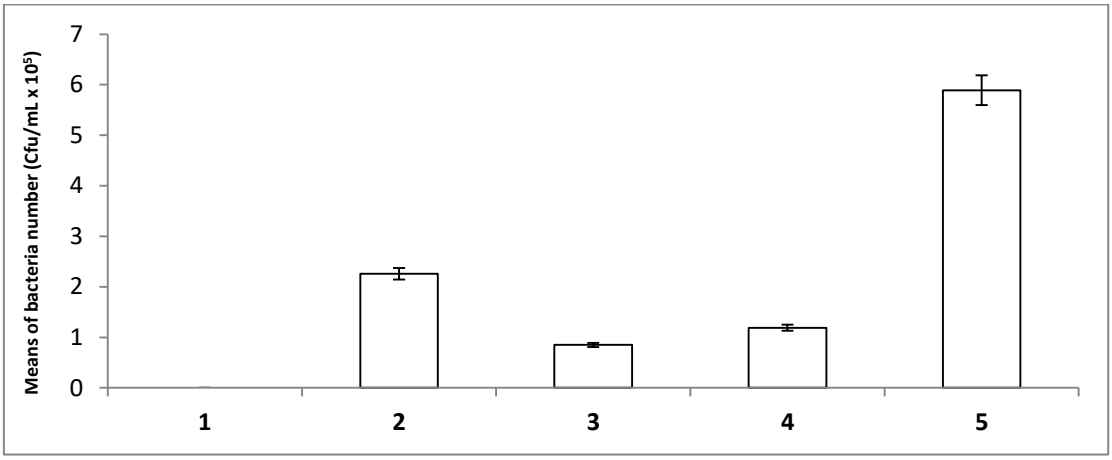

Figure 6. Number of bacteria based on fish length (P value:0.031). Classes: 1=2.8 cm, 2=6.5 cm, 3=7.1 cm, 4=8.5 cm, and 5=9.5 cm.

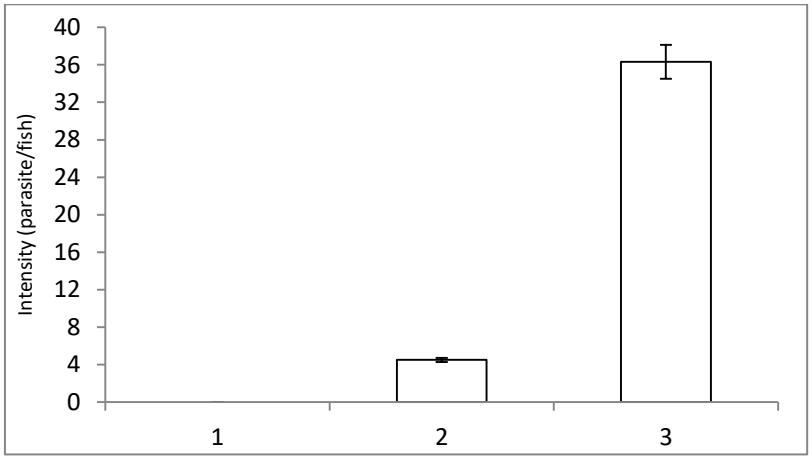

Figure 7. Intensity of *Trichodina* based on fish length interval (P value:0.044). Classes: 1=1-3.5 cm, 2=3.6-7.0 cm, and 3=7.1-9.5 cm.

The calculation of bacteria and parasites number showed a higher number in larger fish (Figure 6 and 7). The number of bacteria was also higher in the heavy infection of parasites (Figure 5). Statistical analysis results using the Kruskal-Wallis test (non-parametric ANOVA) showed significant differences ($P<0.05$) on the mean number of bacteria, based on the parasite infection rates of *Trichodina* (P value=0.039) (Figure 5). The same pattern was obtained in the results of statistical analysis on the number of bacteria based on the length interval of infected fish (P value=0.031) (figure 6) and in the number of parasites based on the length interval of infected fish (P value=0.044) (Figure 7).

Discussion. After identifying all the selected isolates, 8 types of bacteria were obtained all of which were classified as basil bacteria. Khan & Ghosh (2013) stated that most of the genus of *Baccilus* bacteria include aerobic and facultative anaerobic, which can be an indicator that they can live on various niches, explaining their abundance in the aquatic environment. Some researchers reported that the genus of *Baccilus* bacteria is frequently found in the aquaculture industry (Budd 2015). Species of bacteria that belong to the *Bacillus* genus are generally motile and live in a variety of habitats, including the aquatic and sedimentary environments (Nicholson et al 2000).

Some members of the bacterial *Bacillus* genus have been found to cause disease in fish. For example, it has been reported that *Aeromonas* can cause stomach swelling and kidney damage in aquaculture commodities in Ngawi district, Indonesia (Rejeki et al 2016). *Aeromonas* is also a group of bacilli that cause motile aeromonad septicemia (AMS) in some aquaculture commodities, such as the carp and catfish. For instance, *Pseudomonas* is considered responsible for the hemorrhagic septicemia in fish (Foysal et al 2011; Kyeon et al 2016) and *P. damselae* causes several systemic diseases in more than 40 species of fish, including *Sparus aurata* and *L. calcarifer* (Labella et al 2011; Santiago et al 2004; Candan et al 1996).

The present study also demonstrated a relationship between the number of bacteria and the parasite infection rates in certain sizes of fish. Larger fish host a much higher number of bacteria and are subject to higher infection rates. The number of bacteria tended to be higher in the heavy infections with *Trichodina* than in the moderate and light infections. The results of the statistical test analysis showed a significant difference for all the parameters tested. The association between the number of bacterial and the parasites infection rate was investigated and they were found to be strongly associated (Kruskall-Wallis test; $P<0.05$, Figure 5) and the number of bacteria strongly associated with the host length (Kruskall-Wallis test; $P<0.05$, Figure 6). Furthermore, the number of parasites was also strongly associated with the fish length interval (Kruskall-Wallis test; $P<0.05$, Figure 7). This proves that in terms of population abundance, the parasitic infection rates affects the bacterial density. It also shows that the host size influences the number of bacteria and the number of parasites. Haenen et al (2014)

stated that vulnerability occurs in a fish culture when a relationship exists between bacteria and other microorganisms or other variables like host size (in terms of both length and weight). Simultaneous infections are common in fish, therefore interactions between pathogenic microorganisms also occur frequently and have unexpected impacts (Kotob et al 2016).

This study's result can provide a better understanding about the relation of bacterial infections and their association with ciliated trichodinid in the *L. calcarifer* aquaculture. Hahn & Hofle (2001) reported a potential relationship between the abundances of bacteria and protozoan parasites and reveal their relationship with the *L. calcarifer* mortality. Furthermore, the possibility of disease outbreak caused by protozoan and bacterial infection in aquatic fish farm has also been reviewed (Declercq et al 2013). After all, bacteria and protozoan ciliated are the most abundant microorganisms found in the aquatic environment (Lom & Dykova 1992).

Previous studies' results converge with this work, for example concerning the bacterial density showing that bacteria can be transmitted by parasites, promoting the co-infection (Sun et al 2009), and the possibility to reduce it in vital organs such as gills, through the treatment of *Trichodina* infection in fish (Xu et al 2015). Understanding the relation between the co-occurrence of bacteria and protozoan parasite can support alternative management practices in the fish farming and minimising the economic losses (Gomes et al 2019).

The current investigation indicates a higher risk of bacterial infection in larger fish, mediated by the relationship between the pathogens and parasites (*Trichodina*) in *L. calcarifer*. Although not well understood, the disease presence in the aquatic environment is due to a combination of the agents' interaction, the level of host immune resistance and important environmental parameters (Balebona et al 1998). The combination of several important environmental variables is considered responsible for the existence of various diseases and of their agents (Vezzuli et al 2002).

The variation in the level of pathogenic infections in fish can be caused by several factors such as the size and origin of the host, the interferences with other microorganisms, the biological defense of the host, the environmental changes and the season (Mizuno et al 2016).

Conclusions. The present study concluded that there are 8 species of *Bacillus* genus bacteria that have been identified from mucus and gills of infected *L. calcarifer*. Its findings demonstrate a relationship of the bacteria density with the parasites infection rates and with the fish size. The study also provided an initial report on the possible interaction between the occurrence of bacteria with the parasite infection rate on *L. calcarifer* from Indonesia, especially in fish gills and mucus.

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