Malaysian Journal of Microbiology, Vol 15(3) 2019, pp. 237-244
DOI: http://dx.doi.org/10.21161/mjm.180230

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Identification of pathogenic bacteria on the salted fish *Lutjanus vivanus* in Sorong City of West Papua

Sukmawati[1*], Fatimah Hardianti[1], Sipriyadi[2] and Isna Rasdianah Aziz[3]

[1]Fisheries Product Processing, Faculty of Fisheries, Universitas Muhammadiyah Sorong, Sorong city West Papua, 9841 Indonesia.
[2]Department of Biology, Faculty of Mathematic and Natural Sciences, Universitas Bengkulu, Indonesia.
[3]Department of Biology, Universitas Islam Negeri Alauddin Makassar, Indonesia.
Email: sukmawati.sw91@gmail.com

Received 5 September 2018; Received in revised form 21 November 2018; Accepted 23 November 2018

ABSTRACT

Aims: Fisheries-based food is needed by humans as a protein source, one of which is salted fish *Lutjanus vivanus*. Market demand for this product in Indonesia is quite high. The aim of the study was to identify pathogenic bacteria in salted fish *L. vivanus*.
Methodology and results: The method used in this study was descriptive method including pathogenic test, bacterial genomic DNA isolation based on 16 sRNA gene using prokaryotic specific primers, namely 63f forward primer (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse 1387r primer (5′-GGG CGG WGT GTA CAA GGC-3′). The results of this study indicated pathogenic bacteria in salted fish *L. vivanus* with pathogenic activity of α hemolytic and β hemolytic. The bacteria were identified as *Serratia marcescens* strain ZK2 16S for isolate KS and *Bacillus altitudinis* strains A-19 16S for isolate LG.
Conclusion, significance and impact of study: In salted fish *L. vivanus* in the Sorong city, West Papua, it was obtained *Serratia marcescens* strain ZK2 16S with total hemolytic activity and *B. altitudinis* A-19 16S strain with partial hemolytic activity.

Keywords: Lutjanus vivanus, pathogenic bacteria, West Papua

INTRODUCTION

Fisheries-based food is needed by humans as a source of protein. Protein is very important because it contains amino acids that are close to the amino acid composition needed by humans, thus its utilization is more efficient because it is easy to digest (Bahri *et al.*, 2005).

One of the fishery products is salted fish. Salted fish is divided into several types include dried and wet salted fish. Salted fish products can come from various types of fish such as snapper, anchovy, and other types of fish (Salosa, 2013; Muthiadin *et al.*, 2017). Salted fish in various places, particularly Indonesia, are in great demand by the community, yet food safety from these products still needs to be studied in detail.

Sorong City, West Papua, Indonesia is one of the producers of salted fish *Lutjanus vivanus*, this is because most of the population in the city is fishermen, and also supported by favorable geographical location (BPS, 2016). Market demand for *L. vivanus* with the category of dried salted fish in Indonesia is quite high. Salted fish from Sorong city are distributed outside Papua such as Bitung Island, Surabaya, even to Jakarta (Kakisina, 2018).

Microbial total plate count in salted fish *L. vivanus* in the Sorong city was analyzed where sample LG had a number of colonies of 2.36×10^7 cfu/g, sample KB had a number of colonies of 1.84×10^7 - 5.9×10^7 CFU/g (Sukmawati and Hardianti, 2018), and sample KS was 2.06×10^7 - 6.7×10^7 CFU/g, the total number of plates (ALT) exceeds the maximum Indonesian national standard (BSNI, 2009).

Based on the previous description, further research is needed on the identification of pathogenic bacteria in the salted fish *L. vivanus* as information to the public regarding the safety of food consumed, particularly pathogenic contamination. The aim of the study was to identify pathogenic bacteria in salted fish *L. vivanus.*

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 15(3) 2019, pp. 237-244
DOI: http://dx.doi.org/10.21161/mjm.180230

MATERIALS AND METHODS

Isolation

Microbial isolation and total plate count calculation have been carried out by (Sukmawati and Hardianti, 2018). Sample came from Remu market in Sorong city, West Papua. The sample was salted fish *L. vivanus* and coded as KS, LG, and KB. The samples were only from Remu market, which is the central of food supplier and the central of food distributor. Then it distributed to other markets, both traditional markets and modern markets in West Papua region (Reyinaldi, 2017). The samples were taken three times at different times. First phase samples were taken on February 13, 2018 and were coded KS, LG and KB. Second phase samples were on February 27, 2018 were coded TA, TB, and TC. Third phase samples were on March 13, 2018 and were coded PM, PN, and PO. The samples used tightly sealed plastic packaging and were taken from three different points.

Pathogenicity Test

Hemolysis assay or pathogenic test was performed on phase I samples: KS, KB, LG, phase II samples: TA, TB, TC, phase III samples: PM, PN, PO by using Blood agar base with a dose of 40 g/1,000 mL and 5% sterile goat blood. Pathogenicity test is characterized by α hemolysis, β hemolysis, and ɣ hemolysis. Furthermore, the only KS sample and LG sample were identified because the KS sample showed the largest clear zone and represented β hemolysis activity, and the LG sample represented samples that had hemolytic alpha activity.

Bacterial Genomic DNA Isolation (Modified from Sambrook and Russell, 2001)

A total of 1.5 mL of bacterial culture was put into eppendorf and centrifuged at 8000 rpm for 10 min, STE buffer (composited with 0.3 M sucrose; 25 mM Tris-HCL; 25 mM EDTA. 2Na pH 8) used to wash the formed pellet, then centrifuged for 10 min at 8000 rpm. Pellet washing is carried out three time repeatedly.

Furthermore, buffer STE 200 µL and 45 mL lysozyme (20 mg/mL) was added to the pellet then mix and reverse gently. To form protoplast, it then incubated at 55 °C for 60 min. About 20 µL of proteinase-K (20 mg/mL) was added to the mixture and incubated at 55 °C for 1 h. Subsequently, 400 µL of 10% CTAB was added in a solution of 0.7 M NaCl then incubated at 65 °C for half a h. A volume of the phenol:chloroform (25:24) was added into the solution and centrifuged at 12000 rpm for 10 min.

The clear phase formed is then transferred to the new tube. Isopropanol volume was added 0.6 times also 20 µL sodium acetate, the incubation was carried out at -20ºC overnight and centrifuged at 12000 rpm for 10 min. The supernatant was removed while 1 mL pellets was washed using 70% alcohol. DNA was dried for 1 h to remove alcohol and then dissolved in 50 µL sterile ddH_2O, and the results of DNA isolation was stored at 4 °C or –20 °C.

PCR of bacterial 16S rRNA gene

The 16S rRNA gene from genomic DNA was amplified by using PCR using specific primers of prokaryotes (Marchesi *et al*., 1998), namely forward primer 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse primer 1387r (5′-GGG CGG WGT GTA CAA GGC-3′). The composition of the PCR reaction consisted of 0.5 µL La Taq DNA polymerase enzyme, 2× GC 25 µL buffer, 8 µL dNTP mixture, with each primer (10 pmol) 1.5 µl, ddH_2O 9.5 µL, and 4 µL DNA template. PCR conditions used were pre-denaturation (94 °C, 4 min), denaturation (94 °C, 45 sec), annealing (55 °C, 1 min), elongation (72 °C, 1 min 10 sec), and post-PCR (72 °C, 7 min) with a total of 30 cycles.

DNA separation of PCR products was carried out on mini-gel electrophoresis machine using 1% agarose at 75 volts for 45 min. DNA visualization was carried out using UV transilluminator using Ethidium Bromide (EtBr) dye.

The raw sequencing data was trimmed using the ChromasPro version 1.5. Data were performed with BLAST with the genome data registered by the NCBI/National Center for Biotechnology Information.

Some sequences of blast results which the closest species and type strains of each species are were taken from GenBank data at NCBI. Furthermore, the data were re-analyzed by aligning the sequence using the MEGA 6.1 program (Tamura *et al*., 2013) and phylogenetic tree was constructed to show the relationship of isolate Xyl_22 with actinomycetes and other non-actinomycetes using the Neighbor Joining Tree method with a bootstrap of 1000 (Felsenstein, 1985).

RESULTS AND DISCUSSION

Hemolysis assay in samples phase I (KS, KB, and LG), samples phase II (TA, TB, and TC), samples phase III (PM, PN, and PO) showed a positive reaction to pathogens bacteria (Figure 1).

Sample LG and KB showed α hemolysis activity. This hemolysis activity is weak or is usually called partial hemolysis. The hemolysis shows a decrease in hemoglobin of red blood cells for methemoglobin around the colony in the medium, yet the cell membrane is still intact thus partial lysis or weak hemolysis occurs (Harshman and Sugg, 1985; Dybwad *et al*., 2012; Oliveira *et al*., 2014). Since the characteristics and hemolytic activity of sample LG and KB show the same reaction, thus sample LG was further identified because it represents the sample KB and other samples. While the sample KS showed the total hemolytic activity or β hemolysis. It represented the sample TC, sample PN, and sample PO. β hemolysis is defined as the lysis of all red blood cells, which is characterized by forming a clear zone approaching the color and transparency of basic media (Buhnik-Rosenblau *et al*., 2013; Exeni *et al*., 2018).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 15(3) 2019, pp. 237-244
DOI: http://dx.doi.org/10.21161/mjm.180230

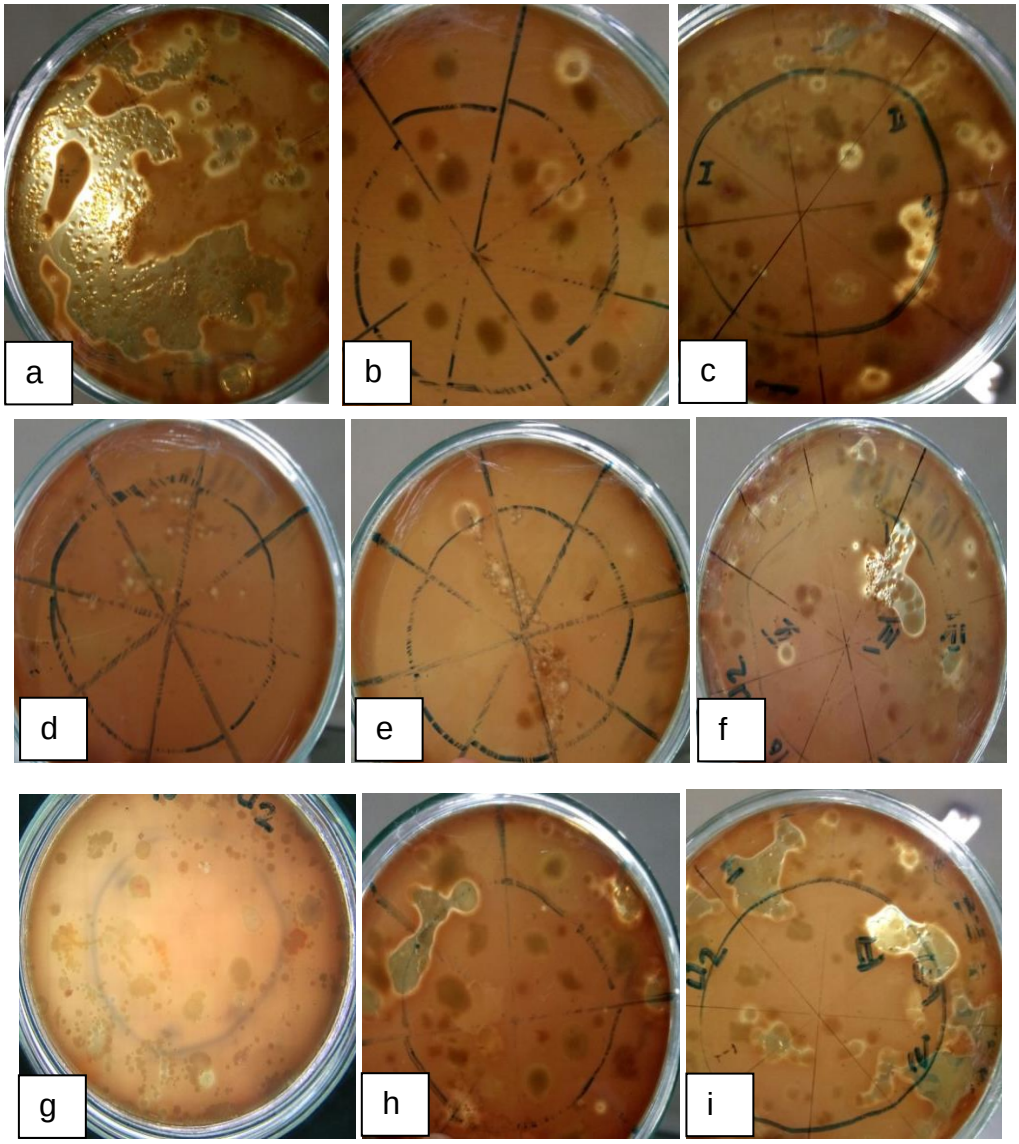

Figure 1: Hemolytic activity on samples; Phase I: a. KS (β hemolysis), b. KB (α hemolysis), c. LG (α hemolysis); Phase II: d. TA (α hemolysis), e. TB (α hemolysis), f. TC (β hemolysis); Phase III: g. PM (α hemolysis), h. PN (β hemolysis), and i. PO (β hemolysis).

Amplification of Genomic DNA in sample KS and LG by PCR method using primers 63f and 1387r produced DNA fragments measuring around 1300 pb (Figure 2). PCR amplification on 16S rRNA gene using primer 63f and 1387r on KS and LG samples had approximately 1300 bp.

Bacterial identification of sample KS and LG showed that isolate KS and LG were in one group or outer group with other bacteria, construction of phylogenetic tree were based on Neighbor Joining Tree method with 1000× bootstrap value. The sample KS was known as *Serratia marcescens* strain ZK2 16S ribosomal RNA gene with partial sequences of 99% (Table 1 and Figure 3). While the sample LG was be found as *Bacillus altitudinis* strain A-19 (Table 2 and Figure 4).

Serratia marcescens is one of the opportunistic pathogenic bacteria belongs to family Enterobacteriaceae which are motile, facultative anaerobes, and have native habitats in the soil, and water (Kurz *et al*., 2003). Facultative anaerobic trait of *Serratia marcescens* is evidenced by hemolytic activity, namely β hemolysis (Figure 1). Characteristic of facultative anaerobic *Serratia marcescens* is supported by packaged salted fish used by distributors, which is tightly closed plastic.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 15(3) 2019, pp. 237-244
DOI: http://dx.doi.org/10.21161/mjm.180230

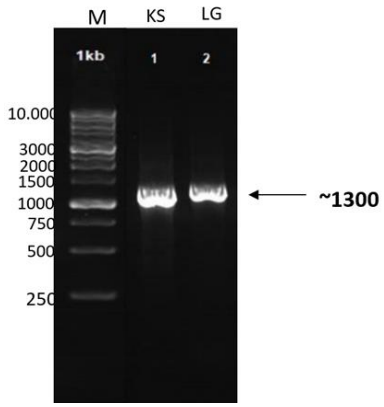

Figure 2: PCR amplification of 16S rRNA gene using 63f primer and primer 1387r; M= marker 1 Kb ladder; sample KS and LG= PCR product of bacterial samples.

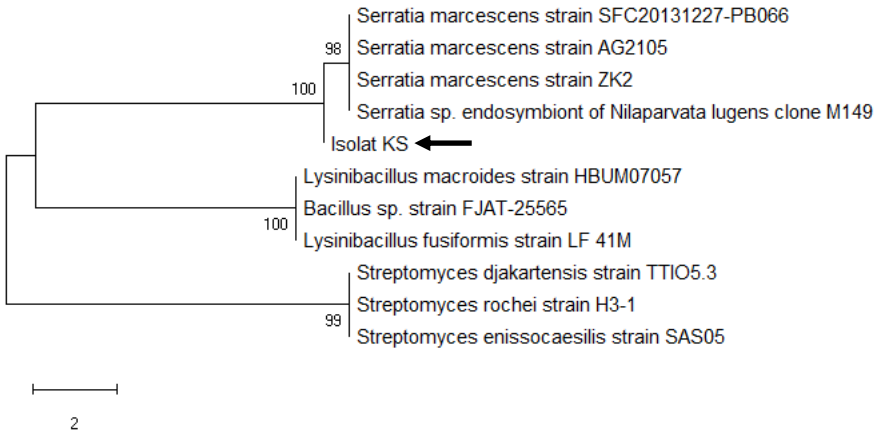

Figure 3: Phylogenetic trees that describe the relationship of isolate KS (arrow) to other bacteria in one group or outer. Construction of phylogenetic tree was based on the Neighbor Joining Tree method with 1000× bootstrap.

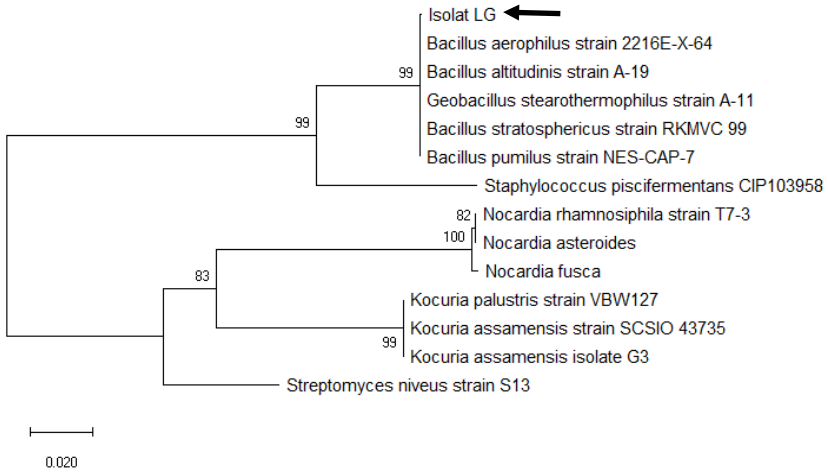

Figure 4: Phylogenetic trees that describe the relationship of isolate LG (arrow) to other bacteria in one group or outer. Construction of phylogenetic tree was based on the Neighbor Joining Tree method with 1000× bootstrap.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 15(3) 2019, pp. 237-244
DOI: http://dx.doi.org/10.21161/mjm.180230

Table 1: Alignment of 16S rRNA gene sequences of isolate KS on available data at NCBI (BLASTX).

Description	Max score	Total score	Query cover	E value	Identity	Access number
Serratia marcescens strain ZK2 16S ribosomal RNA gene, partial sequence	2204	15351	100%	0.0	99%	CP029715.1
Serratia marcescens strain AR-0131 complete genome	2204	15351	100%	0.0	99%	CP029715.1
Serratia marcescens strain SFC20131227-PB066 16S ribosomal RNA gene, partial sequence	2204	2204	100%	0.0	99%	KY992555.1
Serratia marcescens strain AG-2105 16S ribosomal RNA gene, partial sequence	2204	2204	100%	0.0	99%	KY379049.1
Serratia marcescens sp. endosymbiont of Nilaparvata lugens clone M149 16S ribosomal RNA gene, partial sequence	2194	2194	100%	0.0	99%	GU124496.1

Table 2: Alignment of 16S rRNA gene sequences of isolate LG on available data at NCBI (BLASTX).

Description	Max score	Total score	Query cover	E value	Identity	Access number
Bacillus altitudinis strain A-19 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	MH718834.1
Geobacillus stearothermophilus strain A-11 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	MH717377.1
Bacillus stratosphericus strain RKMVC 99 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	MF939145.1
Bacillus aerophilus strain 2216E-X-64 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	MF594129.1
Bacillus pumilus strain NES-CAP-7 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	MF079287.1

Previous studies showed that *Serratia marcescens*, *Streptococcus pyogenes* produce hemolysin under facultative anaerobic conditions (Sylvetsky *et al.*, 2002; Kowalski *et al*., 2003). This has also been proven by the results of Murdoch *et al*. (2011) that *Serratia marcescens* is a Gram-negative bacteria and has bacillary-shaped cells (Arakawa *et al*., 2000; Jo *et al*., 2008). *Serratia marcescens* can cause nosocomial infections, sinusitis, lung abscesses, peritonitis, endocarditis, meningitis, empyema, septic arthritis if the bacterium enters the blood flow and respiratory system (Su *et al*., 2003; Bakkiyaraj *et al.*, 2012).

While the identification of isolate LG showed that the relationship between isolate LG and other bacteria was in one group or outer group. Construction of phylogenetic tree was based on the Neighbor Joining Tree method with 1000× bootstrap and it was obtained *Bacillus altitudinis strain* A-19 16S ribosomal RNA gene with partial sequence 99%. *Bacillus altitudinis* are Gram-negative bacteria and also showed pathogenicity. However, the nature of the pathogen was weak (α hemolysis). According the results of the hemolysis assay, *Bacillus altitudinis* strains AB4 and AB6 were also identified as pathogenic bacteria causing rotten apple and peer. Based on its pathogenicity (Elbanna *et al.*, 2014), the strain is a new finding.

Pathogenic bacteria that contaminate salted fish *Lutjanus vivanus* in the Sorong city, West Papua could come from environmental conditions that are not hygienic or not in accordance with operational standards. In addition, it could also be polluted during the processing process, both from water sources, processing facilities, and packaging as well as transportation process from the processing place to the marketing place. Salosa (2013); Sukmawati and Hardianti (2018) also had revealed that the cleanliness of the processing of fishery products and their distribution in the city of Sorong has not met the recommended standards.

The hygiene of the processing place based on operational standards is very important in order to maintain the quality of preserved processed products such as meat and other processed products (Sukmawati,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

2018; Sukmawati *et al.*, 2018) as well as fishery products. All food must be safe for consumption, safe from pathogenic microbial contamination, the number of microbes does not exceed the recommended maximum limit, free from harmful physical substances, and safe from chemical preservatives that are not recommended.

CONCLUSION

Sample KS was identified as *Serratia marcescens* ZK2 16S ribosomal RNA gene, with partial sequence of 99% and had β-hemolysis activity, while sample LG was identified as *Bacillus altitudinis* A-19 16S ribosomal RNA gene strain with partial sequence of 99% and had α-hemolysis. Therefore, we conclude that salted fish *Lutjanus vivanus* is not suitable for consumption.

ACKNOWLEDGEMENTS

This study is a research funded by the Ministry of Research, Technology and Higher Education (KEMENRISTEK-DIKTI) through the beginner lecturer research grant (PDP) of funding program 2018.

REFERENCES

Arakawa, Y., Shibata, N., Shibayama, K., Kurokawa, H., Yagi, T., Fujiwara, H. and Goto, M. (2000). Convenient test for screening metallo-beta-lactamase-producing Gram-negative bacteria by using thiol compounds. *Journal of Clinical Microbiology* **38(1), 40-43.**

Bahri, S., Masbulan, E. and Kusumaningsih, A. (2005). Preproduction process as an important factor in producing livestock products that are safe for humans. *Jurnal Litbang Pertanian* **24(1), 27-35.**

Bakkiyaraj, D., Sivasankar, C. and Pandian, S. K. (2012). Inhibition of quorum sensing regulated biofilm formation in *Serratia marcescens* causing nosocomial infections. *Bioorganic and Medicinal Chemistry Letters* **22(9), 3089-3094.**

BPS. (2016). The number of fisheries, Fishermen, and production households according to the district in the city of Sorong, in 2015. https://sorongkota.bps.go.id. [Retrieved on August 12, 2018].

BSNI. (2009). Maximum limit of microbial contamination in food. Indonesian National Standarts. Jakarta.

Buhnik-Rosenblau, K., Matsko-Efimov, V., Danin-Poleg, Y., Franz, C. M. A. P., Klein, G. and Kashi, Y. (2013). Biodiversity of *Enterococcus faecalis* based on genomic typing. *International Journal of Food Microbiology* **165(1), 27-34.**

Dybwad, M., Granum, P. E., Bruheim, P. P. and Blatnya, J. M. (2012). Characterization of airborne bacteria at an underground subway station. *Applied and Environmental Microbiology* **AEM.07212, 1-51.**

Elbanna, K., Elnaggar, S. and Bakeer, A. (2014). Characterization of *Bacillus altitudinis* as a new causative agent of bacterial soft rot. *Journal of Phytopathology* **162(11-12), 712-722.**

Exeni, R. A., Fernandez-Brando, R. J., Santiago, A. P., Fiorentino, G. A., Exeni, A. M., Ramos, M. V. and Palermo, M. S. (2018). Phatogenic role of inflammatory response during shiga toxin-assosiated hemolytic uremic syndrome (HUS). *Pediatric Nephrology* **2018, 1-15.**

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39(4), 783-791.**

Harshman, S. and Sugg, N. (1985). Effect of calcium ions on staphylococcal alpha-toxin-induced hemolysis of rabbit erythrocytes. *Infection and Immunity* **47(1), 37-40.**

Jo, G. H., Jung, W. J., Kuk, J. H., Oh, K. T., Kim, Y. J. and Park, R. D. (2008). Screening of protease-producing *Serratia marcescens* FS-3 and its application to deproteinization of crab shell waste for chitin extraction. *Carbohydrate Polymers* **74(3), 504-508.**

Kakisina, E. B. (2018). Fishermen kofiau Raja Ampat salted fish production. Sorong: Antaranews Papua Barat https://papuabarat.antaranews.com/. [Retrieved on August 30, 2018].

Kowalski, R. P., Dhaliwal, D. K., Karenchak, L. M., Romanowski, E. G., Mah, F. S., Ritterband, D. C. and Gordon, Y. J. (2003). Gatifloxacin and moxifloxacin: An *in vitro* susceptibility comparison to levofloxacin, ciprofloxacin, and ofloxacin using bacterial keratitis isolates. *American Journal of Ophthalmology* **136(3), 500-505.**

Kurz, C. L., Chauvet, S., Andrès, E., Aurouze, M., Vallet, I., Michel, G. P. F., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J. A., Finlay, B. B., Gorvel, J. P., Ferrandon, D. and Ewbank, J. J. (2003). Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. *The EMBO Journal* **22(7), 1451-1460.**

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. and Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology* **64(2), 795-799.**

Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E. and Coulthurst, S. J. (2011). The opportunistic pathogen *Serratia marcescens* utilizes type VI secretion to target bacterial competitors. *Journal of Bacteriology* **193(31), 6057-6069.**

Muthiadin, C., Aziz, I. R., and Andriyani, A. A. (2017). *Awaous melanocephalus*: Ikan native species dari Sulawesi Barat (sebuah review). *Prosiding Seminar Nasional Biology for Life* **3, 55-59.**

Oliveira, T. L., Caetano, A. Z., Belem, J. M., Klemz, B. C. and Pinheiro, M. M. (2014). Interferon-α induced psoriatic arthritis and autoimmune hemolytic anemia during chronic hepatitis C treatment. *Acta Reumatologica Portuguesa* **39, 327-330.**

Reiynaldi. (2017). Papua news. https://kabarpapua.co/pasar-sentral-sorong-layak-jadi pasar-wisata/. [Retrieved on August 15, 2018].

Salosa, Y. Y. (2013). Examination of formalin and salt concentrations and total bacteria in the mackerel salted fish from Sarmi district, Papua Province. *Depik* **2(1), 10-15.**

Sambrook, J. F. and Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual. 3rd Edn. Cold Spring Harbor Laboratory Press. New York.

Su, L. H., Ou, J. T., Leu, H. S., Chiang, P. C., Chiu, Y. P., Chia, J. H., Kuo, A. J., Chiu, C. H., Chu, C., Wu, T. L., Sun, C. F., Riley, T. V. and Chang, B. J. (2003). Extended epidemic of nosocomial urinary tract infections caused by *Serratia marcescens*. *Journal of Clinical Microbiology* **41(10), 4726-4732.**

Sukmawati. (2018). Total microbial plates on beef and beef offal. *Bioscience* **2(1), 22-28.**

Sukmawati and Hardianti, F. (2018). Analysis of microbial total plate count (TPC) in snapper salted fish in the city of Sorong, West Papua. *Jurnal Biodjati* **3(1), 72-78.**

Sukmawati, Ratna and Fahrizal, A. (2018). Analysis of microbial contamination in broiler chicken meat in the city of Makassar. *Scripta Biologica* **5(1), 51-53.**

Sylvetsky, N., Raveh, D., Schlesinger, Y., Rudensky, B. and Yinnon, A. M. (2002). Bacteremia due to beta-hemolytic *Streptococcus* group G: Increasing incidence and clinical characteristics of patients. *The American Journal of Medicine* **112(8), 622-626.**

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30(12), 2725-2729.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538