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Physiological Profiling and Microorganism Community Analysis of Cirebon Shrimp Paste Fermentation "Terasi" using BIOLOG™ EcoPlate

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Terasi or shrimp paste is an Indonesian traditional seasoning made from fermented small shrimp or krill. Different indigenous microorganism community exhibit different physiological function due to lack standard in its materials and processing. This study aimed to determine physiological profiles and microorganism community in Cirebon shrimp paste fermentation. BIOLOG™ EcoPlate was used to obtain microbial physiological function of the krill and 2-months old shrimp paste. Microorganisms were later isolated from EcoPlate substrate to determine its community structure. Average Well Color Development (AWCD) from krill was thirty times higher than shrimp paste. Interestingly, this study revealed a shift of carbon source utilization at day-28 of fermentation from amino acid and polymer to phenolic compound. In addition, AWCD index increased in accordance with increased of microorganism community complexity at day-28. Within 56 days of fermentation there was a slightly increase in water, fat, and carbohydrate content. In contrast, there was decrease in protein, ash content, and acidity level from neutral to acid, with salinity level resulted in between 16.26% to 21.42%. We conclude that there is a change of microorganism community within shrimp paste fermentation corresponding to metabolism activity which affects the product quality.

Key words: BIOLOG™ EcoPlate, microbial community, physiology, shrimp paste

Terasi udang merupakan salah satu contoh penyedap rasa alami dari Indonesia dengan bahan baku udang rebon yang difermentasi. Tahap pembuatan serta bahan baku yang tidak sama membuat komunitas mikroorganisme serta aktivitas metabolisme yang terlibat akan berbeda. Dalam penelitian ini akan ditentukan profil fisiologi dan komunitas mikroorganisme selama proses fermentasi terasi udang dari Cirebon, Jawa Barat. Analisis fungsi fisiologi dilakukan dengan menggunakan BIOLOG™ EcoPlate pada sampel udang rebon dan terasi setiap 2 minggu selama 2 bulan fermentasi. Mikroorganisme diisolasi dari substrat BIOLOG™ EcoPlate untuk menentukan struktur komunitasnya. Nilai *Average Well Color Development* (AWCD) BIOLOG™ EcoPlate dari sampel udang rebon lebih tinggi 30 kali daripada sampel terasi udang. Selain itu, terlihat pergantian penggunaan kelompok substrat dari polimer dan asam amino menjadi senyawa fenolik pada terasi berumur 28 hari. Nilai AWCD pada sampel terasi mengalami penurunan pada hari ke-28 dan kembali mengalami kenaikan hingga hari ke-56 seiring dengan adanya peningkatan kompleksitas dari komunitas mikroroganisme. Selama 56 hari fermentasi, terjadi sedikit pengingkatan kadar air, lemak, dan karbohidrat. Sebaliknya, kadar protein dan abu mengalami penurunan dan rentang pH dari netral menuju asam. Kadar garam berfluktuatif pada rentang 16,26% hingga 21,42%. Melalui penelitian ini dapat disimpulkan bahwa adanya perubahan komunitas mikroorganisme pada fermentasi terasi udang melalui perbedaan kemampuan penggunaan substrat karbon.

Kata kunci: BIOLOG™ EcoPlate, fungsi fisologis, komunitas mikrooganisme, terasi udang

Terasi or shrimp paste is one of Indonesian traditional fermented product made from shrimp that undergo natural fermentation by the work of indigenous microorganism (Kobayashi *et al.* 2003; Murwani *et al.* 2015). Similar product as food seasoning are also found in many Asian countries, such as China, Malaysia, Bangladesh, and Korea (Kim *et al.* 2014). In Indonesia, shrimp paste produced (by generation to generation) in many coastal areas where source of raw materials such as shrimp, krill, and fish are abundant.

According to Indonesian National Standard (Standar Nasional Indonesia - SNI, 1992), shrimp paste is a food seasoning with distinctive odor from fermentation of shrimp or fish or mixture of both with salt or other ingredients. Exact procedures and ingredients of shrimp paste varies according to region. However, similar step of grinding, drying, and salt addition are always present. Addition of salt prevent spoilage and creating a suitable environment for certain microorganism (Lee *et al.* 2016). Breakdown of protein compounds into amino acids and various chemicals corresponds to umami taste and some antioxidant properties (Murwani *et al.* 2015).

Since fermentation occurred naturally, both functional and non-functional microorganisms might

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contribute in the process (Tamang *et al.* 2016). Different manufacturing process will certainly give unequal results for each product, because physiology of the microorganism are strongly influenced by environment. To date, various microorganisms such as *Bacillus, Micrococcus, Tetragenococcus, Sarcina, Pseudomonas, Sporolactobacilus, Pediococcus, Streptococcus, Halomicrobium,* and *Streptococcus* group are recorded to be found in shrimp paste with different physiological properties such as proteolytic, halophilic, and lactic acid production (Chukeatirote 2016; Kobayashi *et al.* 2003; Sobhi *et al.* 2012; Surono and Hosono 1994).

Microorganism community implies its functional physiology property by its catabolic processes as indicated by its capability to adapt to different metabolic condition (Preston-Mafham, Boddy, and Randerson 2002). The microorganism community level physiological profile can be assessed using BIOLOG™ EcoPlate introduced by Garland and Mills. BIOLOG™ EcoPlate consist of 96-well with 31 different carbon substrates based on redox reaction. Reduction of tetrazolium dye in each well produces color change and are used as an indicator of respiration. The redox reaction are exploited for rapid assessment or characterization of microbial communities (Garland and Mills 1991).

Study on physiological function and dynamics of microorganism community within shrimp paste fermentation has not been reported. Studies on community level of microorganism and its physiology that contribute to determine shrimp paste quality is limited. This study is intended to assess physiological function of microorganism community in shrimp paste fermentation using BIOLOG™ EcoPlate by determining the microbial community dynamics and its physiological activity especially in Cirebon shrimp paste fermentation.

MATERIALS AND METHODS

Sample. Traditionally produced Cirebon shrimp pastes were collected from Samadikun, Cirebon, West Java. According to producer, shrimp or krill were dried for 1 d under the sun, then pounded and dried again for 1 d. Salt and sugar were added using krill : salt : sugar ratio of 5:1:1 (w/w). A 500 mg of mixture were wrapped with waxed paper and then stored at room temperature to undergo fermentation. Shrimp paste was sampled every 14 d for 2 m.

Community Level Physiological Profile. Sample was prepared and inoculated into the BIOLOG™ EcoPlate based on previous method (Wang *et al.* 2007) with some modifications. Briefly, 4 g of crushed shrimp paste or krill was suspended in 36 mL of saline phosphate buffer solution or PBS; pH 7.2. Homogenate was filtered using sterile gauze to remove large particles and was diluted by introducing 4 mL of homogenate into 36 mL of PBS. A 150 µL of homogenate from 10^{-3} dilution was inoculated into each BIOLOG™ EcoPlate well. Incubation was performed at 37 °C for 168 h. Optical density was measured every 24 hours at 590 nm and 750 nm. Measurements at 590 nm were to detect color changes due to formazan formation and turbidity, while measurements at 750 nm were for turbidity correction. Negative value was corrected to zero. Mean of each substrate density value from three repetitions was used for further calculations. The Average Well Color Development (AWCD) value for each observation time was calculated according to following formula (Garland and Mills 1991):

$$AWCD(t) = \left[\sum_{i=1}^{31}(C(t) - R(t))\right]/31$$

Information:
C (t) = optical density value of i substrate at a given time; R (t) = optical density value of control at a given time; i = number of substrates.

Total plate count. Krill and shrimp paste homogenate were inoculated on Nutrient Agar (NA) media and incubated at 37 °C for 24 h. Enumeration was performed to obtain total plate count number expressed in colony forming unit per mL (CFU mL^{-1}).

Isolation and Characterization of Microorganism. Microorganism isolation was performed from each well of EcoPlate with positive value after 168 h. A 100 µL of sample composite was inoculated to Nutrient Agar (NA) media and incubated at 37 °C for 24 h. Single isolate is characterized based on colony morphology and microscopic observations by means of Gram staining according to previous method (Prescott, Klein, and Harley 2002).

Statistical Analysis. Multivariate analysis was performed by Principle Component Analysis (PCA) using XLSTAT. Analysis was performed to differentiate krill and shrimp paste, physiological function and microorganism community during fermentation. The PCA was determined based on Pearson type, where number of microorganism, optical density value of each BIOLOG™ EcoPlate substrates, and isolates obtained were used as variables.

Measurement of salinity and acidity. Salinity was measured by argentometric according to Mohr method (Hong, Kim, and Czae 2010) with some modifications. Briefly, 4 g sample was added to 36 mL sterile water and filtered by Whatman paper no. 1 before titration. Meanwhile, acidity level was measured using a calibrated pH-meter.

Proximate Analysis. Proximate analysis was conducted to obtain carbohydrate, protein, fat, ash, and lipid content from samples. It was performed in accordance to SNI 01-2891-1992.

RESULTS

Samples obtained from krill and traditionally fermented shrimp paste at five time-points were assessed by BIOLOG™ EcoPlate, total plate count, and chemical characteristic. Samples were first assessed by BIOLOG™ EcoPlate to obtain physiological function at community level. Total plate count performed to verify microorganism density that might contribute to turbidity changes in EcoPlate. Microorganism isolation from each BIOLOG™ EcoPlate utilized substrates was performed for community analysis.

Average Well Color Development (AWCD) value represented metabolic activity of microorganism community from metabolized substrates. The AWCD of krill sample after 168 h incubation showed the highest value among all samples at the value of 0.404, thirty times higher than other samples (Fig 1A). Meanwhile, AWCD value of shrimp paste are lower at the value of 0.013; 0.007; 0.004 for day-0, -14, and -28 of shrimp paste with slight increase at day-42 and 56 at the value of 0.008 and 0.009 respectively (Fig 1B). In general, the result implied that microbial community of shrimp paste fermentation had lower metabolic activity than that of krill, whereas the slight increase at day-42 indicate changes in the fermentation process.

The numbers of microorganism involved during fermentation were determined by culturing the microorganism on Nutrient Agar (NA) media. Samples were enumerated and expressed in colony forming unit per mL (CFU mL^{-1}). Total plate count of microorganism enumeration in shrimp paste samples were significantly lower than that of microorganism in krill samples (Table 2). This result strongly contributed to Principle Component Analysis (PCA) plot where krill and shrimp paste samples were obviously located at different area (Fig 2). This strongly suggest that krill and shrimp paste samples were significantly different in term of number of microorganism.

Mean substrate utilization on BIOLOG™ EcoPlate showed dynamics of carbon source group utilization during shrimp paste fermentation. Utilization of carbohydrate, polymer, and carboxylic acid from day-0 to -28 showed decreasing pattern, except for amino acid (Fig 3A). Interestingly, amino acid and polymer utilization was not found at day-28 and -42 respectively and were replaced by phenolic utilization. Corresponding to substrate group utilization, microorganism dynamics were also found during fermentation (Fig 3B). The number of isolates found at day-0, -14, -28, -42, and -56 were 8, 8, 7, 8, and 7 isolates respectively. Each isolate were obtained from different carbon source that can be grouped into amino acid (AA), carbohydrate (CH), carboxylic acid (CA), polymer (PO), and phenolic (PH) with total number of isolates for each carbon group were 3, 16, 3, 4, and 3 respectively. AA1, AA2 and CH1 were found in day-0 and -14, while CA1 were always present at day-0 to -28. GM1 and GA5 were found at day-28 and -42 of fermentation, and PO1, PO2, PO3, and PO4 were found in day-14 and -28. As the figure 3 show microbial shifting occurred when CH10, CH11, CH12, PH1, PH2, and PH3 were obtained from carbohydrate and phenolic substrates at day-42 and -56.

PCA plot for physiological function and microorganism community clearly separated the physiological function at day-0, 14, and -28 of fermentation, meanwhile day-42 and -56 of fermentation are adjacent (Fig 4A). In contrast, opposite pattern is found when comparing microorganism community, where day-0, -14, -28 of fermentation are plotted close to each other and distinct from day-42 and -56 (Fig 4B).

As shown by the analysis, physiological activity and microorganism dynamics affect nutritional and chemical characteristic of shrimp paste during fermentation (Table 2). However, the dynamics are not reflected by its acidity or pH level which is generally at neural-acid range. pH level slightly increases at the beginning of fermentation from 7.02 to 7.08 and decreased to 6.74 at the last day of fermentation. Salt content fluctuated in the range of 16.26% to 20.48%. Water content drastically decreased from shrimp sample to day-0, from 86.70% to 41.74%. Nevertheless, there are no significant changes in water content during fermentation process. Fat and carbohydrate content increased from 1.32% and 17.26% to 1.47% and 19.94%, respectively. In contrast, protein and ash content decreased from 12.43% and 27.25% to 10.46% and 25.76%, respectively.

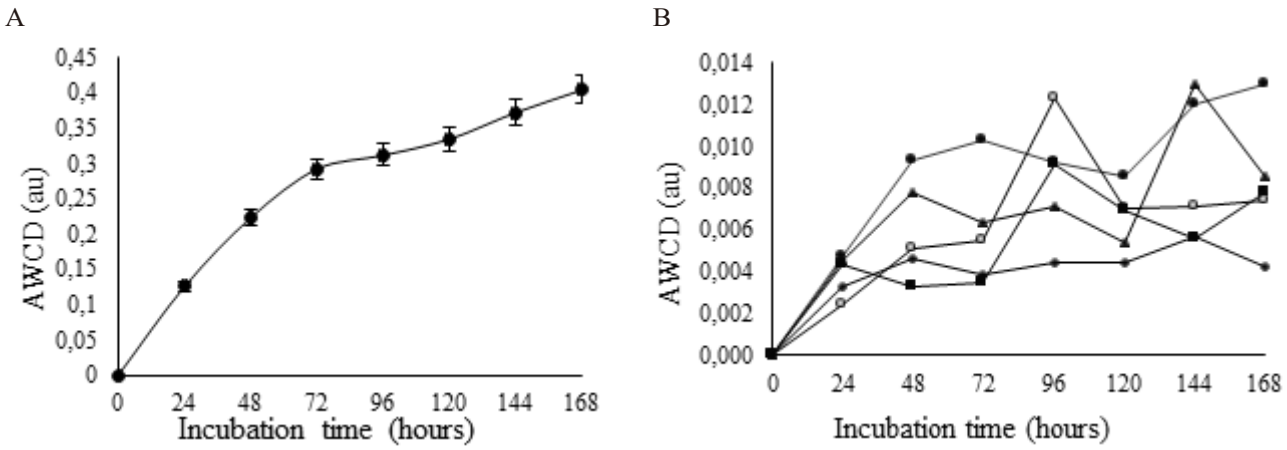

Fig 1 Potential metabolic activity of microorganism community in krill sample (*A*) and shrimp paste (*B*) by AWCD values from BIOLOG™ EcoPlate. Krill and five time-points of shrimp paste fermentation samples were inoculated into each EcoPlate well and measured every 24 hours for 7 d. Based on 168 hours of incubation, krill sample exhibit highest AWCD value among all samples. In general, AWCD value for day-0 (●), -14 (○), -28 (♦), -42 (■) and -56 (▲) of shrimp paste were lower than of krill sample. Error bar on shrimp paste samples were not shown to simplify data visualization.

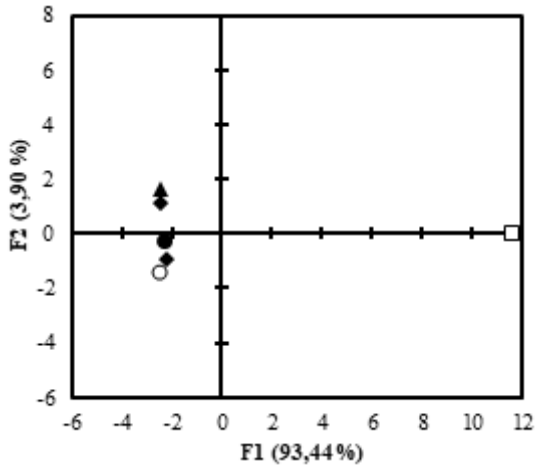

Fig 2 Principle Componenet Analysis (PCA) plot of krill and shrimp paste samples. Analysis was based on total plate count of microorganism on krill and shrimp paste samples isolated grown on Nutriet Agar media. Day-0 (●), -14 (○), -28 (♦), -42 (■) and -56 (▲) of shrimp paste were adjacent to each other and distinct to (□) krill sample.

Table 1 Total plate count enumeration of microorganism on krill and shrimp paste samples during fermentation grown on Nutrient Agar media

Sample	Total plate count (CFU mL^{-1})
Krill	3.7×10^8
Day-0 shrimp paste	2.2×10^4
Day-14 shrimp paste	3.1×10^4
Day-28 shrimp paste	3.0×10^4
Day-42 shrimp paste	4.2×10^4
Day-56 shrimp paste	1.5×10^4

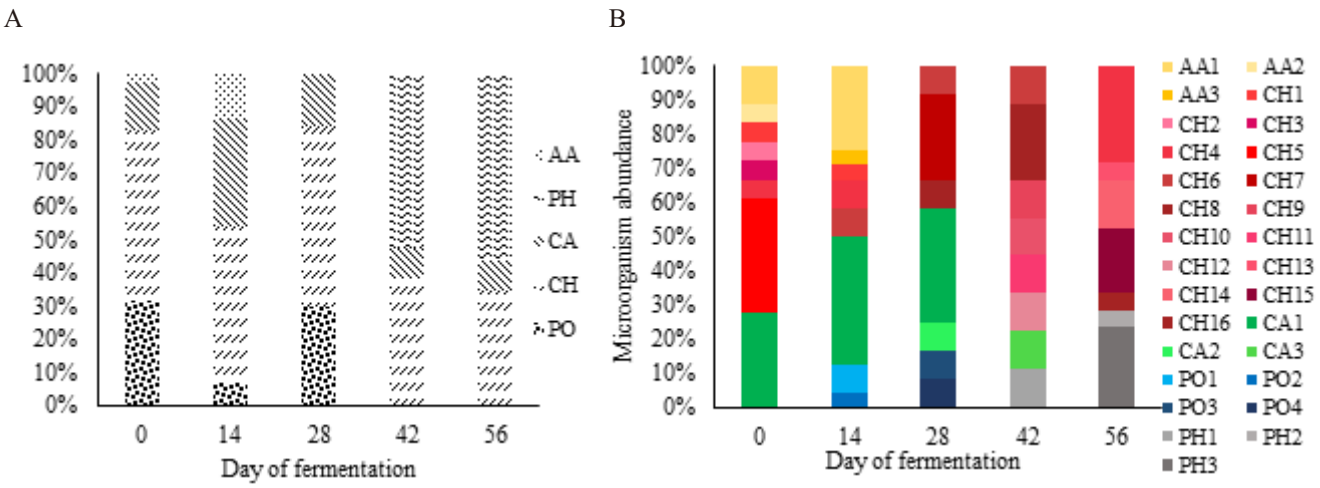

Fig 3 Dynamics of relative carbon source group utilization (*A*) and microorganism abundance (*B*) within 56 d of fermentation. Carbon group utilization data was obtained from relative sum of mean utilization for each carbon group to whole substrate utilized from BIOLOG™ EcoPlate after 16*8 h* incubation. Microorganism abundance shown during fermentation were characterized by colony morphology and Gram staining. Changes of carbon group utilization were shown at day-28 when polymer and amino acid were replaced by phenolic group utilization. Corresponding to carbon group utilization, 3, 16, 3, 4, and 3 isolates used amino acid (AA), carbohydrate (CH), carboxylic acid (CA), polymer (PO), and phenolic (PH) substrates, respectively.

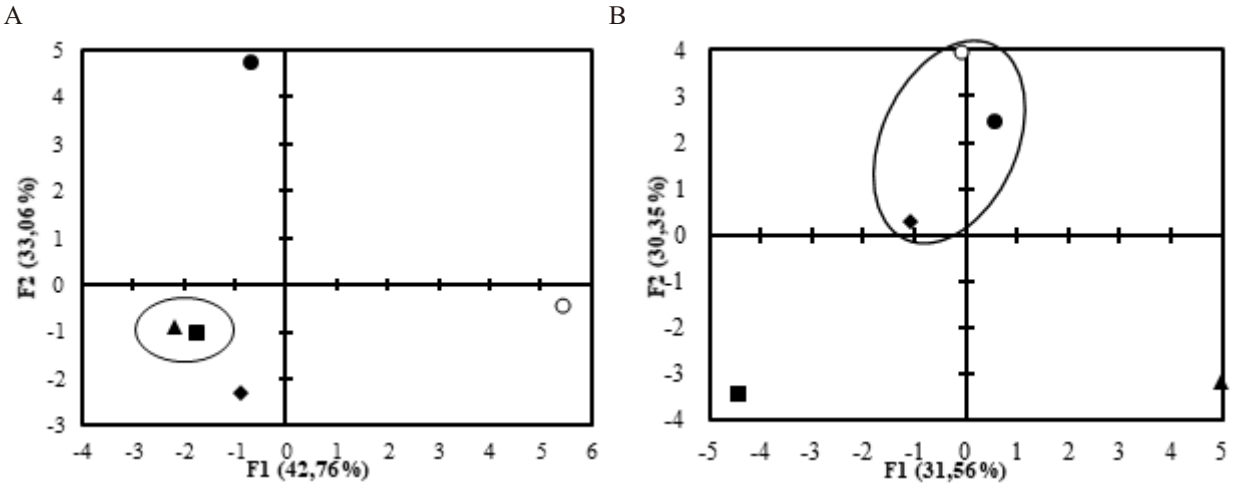

Fig 4 PCA observation plot for (*A*) physiological analysis and (*B*) community analysis on shrimp paste sample within 56 days of fermentation. Day-0 (●), -14 (○), -28 (♦) of fermentation are adjacent in microorganism community, but distinct in physiological function. Meanwhile, day-42 (■) and -56 (▲)of fermentation are adjacent in physiological function, but distinct in microorganism community.

Table 2 Chemical and nutritional characteristics of shrimp paste during fermentation

Sample	pH	Salt (%)	Water (%)	Ash(%)	Protein (%)	Fat(%)	Carbohydrate (%)
Shrimp	nd[1]	nd[1]	86.70	1.83	7.68	0.73	2.93
Drying I	nd[1]	nd[1]	39.72	nd[1]	nd[1]	nd[1]	nd[1]
Drying II	nd[1]	nd[1]	35.36	nd[1]	nd[1]	nd[1]	nd[1]
Day-0	7.02	19.77	41.74	27.25	12.43	1.32	17.26
Day-14	7.08	16.26	41.76	nd[1]	nd[1]	nd[1]	nd[1]
Day-28	6.83	21.41	41.92	24.73	10.34	2.02	20.99
Day-42	6.89	20.48	42.91	nd[1]	nd[1]	nd[1]	nd[1]
Day-56	6.74	20.48	42.73	25.76	10.46	1.47	19.94

[1]nd = not determined

DISCUSSION

BIOLOG™ EcoPlate provides rapid and complex community level profiling on physiological function to characterize microorganism community. The assessment is based on carbon source utilization coupled to tetrazolium dye reduction as an indicator of respiration (Garland and Mills 1991). Average Well Color Development (AWCD) from BIOLOG™ EcoPlate reflect the rate of substrate utilization by microorganism community (Preston-Mafham *et al.* 2002). In addition, inoculum density and different community structure may result in higher absorbance values (Preston-Mafham *et al.* 2002). AWCD value of krill sample was thirty times higher than that of shrimp paste (Fig 1A and 1B) suggests that rate of catabolic activity from krill was higher than shrimp paste. This might be due to the number of microorganism in krill that were significantly higher than that of shrimp paste (Table 1). Drying process in shrimp paste production reduce the water content and implicate towards low number of microorganism. Salt addition also provide suitable condition only for certain microorganism to grow and prevent putrefaction (Anggo *et al.* 2015).

BIOLOG™ EcoPlate can reveals catabolic diversity from samples, in which each microorganism pursue its own substrate utilization pattern, which in turn indicative of the community species diversity. As much as 31 carbon or sole substrate sources are available in EcoPlate and can be further categorized into several substrate group: 10 carbohydrates, 4 polymers, 8 amino acids, 2 phenolics, and 7 carboxylic acids (Deng *et al.* 2011). Based on these groups, microorganism in shrimp paste utilized carbohydrate, polymer, amino and carboxylic acid in higher level in the early 28 days of fermentation. However, amino acid and polymer utilization were replaced by phenolic utilization after 28 days (Fig 2A). The dynamics of substrate utilization corresponds to microorganism dynamics found in each substrate (Fig 2B).

Amino acids utilization was found in the early 28 days of fermentation, which corresponds to the decreasing of protein at day-28. Nevertheless, further fermentation does not alter protein content (Table 2). This suggests that proteolytic microorganism, those possessing protease enzymes to breakdown protein into other peptides, amino acids and other volatile compounds (Khairina *et al.* 2016; Murwani *et al.* 2015), are present in shrimp paste. The amino acids utilized in EcoPlate were L-arginine, L-threonine, L-asparagine, and L-serine. Several isolates, such as AA1 and AA2 are found to utilize L-arginine and L-asparagine at day-0 and -14 of fermentation, respectively. AA1 has circular white opaque colonies with coccus-shaped Gram negative while AA2 has circular white transparent colonies with rod-shaped Gram negative.

Carbohydrates and carboxylic acids utilization, which could be derived from sugar degradation, were always detected throughout fermentation. The use of BIOLOG™ EcoPlate were able to identify changes of carbohydrate used, N-acetyl glucosamine replaced by β-methyl-D-glucoside and D-mannitol at day-42 and -56. It is known that N-acetyl glucosamine is chitin monomer presented in 1,4-β-glycoside bond (Beier and Bertilsson 2013) found in krill. Isolate CA1 was isolated from the well with D-galacturonic-acid at day-0 and -14, pyruvic acid methyl ester at day-14, and tween 80 at day-28. The isolate is characterized by coccus-shaped, Gram positive, white opaque colonies and circular shape. CH10; CH11; and CH12 were found in N-acetyl-glucosamine with following characteristics: circular white opaque colonies with coccus-shape Gram negative; circular white opaque colonies with coccus-shaped Gram positive; irregular cream opaque colonies with coccoid-shaped Gram positive, respectively.

Polymer utilized in EcoPlate were tween-40 and tween-80 at day-0 and -28. Tween-80 and tween-40 are fatty acid derivatives from oleic acid and palmitic acid, respectively (Ilko *et al.* 2015; Takeno *et al.* 2013). Oleic acid and palmitic acid compounds could be a source of carbon in the growth of microorganism through the reaction of β-oxidation. In the other hand, these two fatty acids were dominantly found abundant in krill or shrimp (Li, Sinclair, and Li 2011). The isolates PO1; PO2; PO3; and PO4 were found and characterized by irregular white opaque colonies with rod-shaped Gram negative; irregular white opaque colonies with rod-shaped Gram negative; circular white opaque colonies with coccus-shaped Gram negative; and circular yellow opaque colonies with coccus-shaped Gram negative, respectively.

Phenolic compounds were used in last stage of fermentation and influenced the flavor development (Mcmurrough, Roche, and Cleary 1984) and antioxidant properties of shrimp paste (Sobhi et al. 2012). The phenolic compound of 4-hydroxy-benzoic-acid were used as the main carbon source by the halophile group *Halobacteria* of Archaea (Fairley *et al.*

2002). Utilization of aromatic compounds in the last day of fermentation is in accordance with a study by Wittanalai, Rakariyatham, and Deming (2011) that examined Kapi products from Thailand and found that the compounds involved in the formation of a distinctive aroma were obtained in large amounts after 30 d of fermentation. The isolates PH1; PH2; and PH3 are found at day-56, and characterized by large-sized opaque white colonies with coccus-shaped Gram-negative; circular large white colonies with rod-shaped Gram negative; and large white colonies with coccus-shaped Gram positive, respectively.

PCA plot for functional and community structure clearly separate different sampling time indicative of different physiological function and community structure during shrimp paste production (Fig 4). Microorganism functional characteristic of day-0, -14, and -28 were dispersed and shown to be distinct between each other, but its community characteristics were plotted adjacent to each other indicative of its similarity or closeness. In opposite, microorganism from sample day- 42 and -56 showed close functional characteristics with distinct microorganism community. This suggests that the functional of microorganism community at the beginning of fermentation change more dynamically than at the end of fermentation period. In contrast, microorganism involved in early stage was not drastically changed within 28 d of fermentation. Other causes of differences in PCA plots are due to isolation and identification of isolates derived from EcoPlate which might cause a reduction number of microorganism in isolation medium. Microorganism growth were dependent on media culture (Vieira and Nahas 2005) while EcoPlate has a specific substrate as main carbon source for microorganism to grow which not presented in media.

Chemistry and nutrition parameters of shrimp paste within 2 months of fermentation showed an interesting result. The pH level during early stage of fermentation increase because of the activity of microorganism that metabolize amino and amine compounds to produce NH_3 compounds (Daroonpunt *et al.* 2016). Later, the lower pH level at day-28 to -56 were due to the production of acid compounds, where lactic acid bacteria might contribute in this process, as previously shown Kobayashi *et al.* (2003) and Surono and Hosono (1994). Lactic acid bacteria might be found from carbohydrate utilization microorganism, where they utilize sugar compound and release lactate as metabolite product (Justé *et al.* 2008).

Fluctuating values of salt content might be due to uneven stirring process during shrimp paste making and causes different salt content in packaging. However, above 10% of salinity content is considered to help preservation process (Christianti 2006). Besides, the drastic decrease of water content by means of drying process also contribute in product preservation by inhibiting the growth of undesired microorganism (Anggo *et al.* 2015).

Badan Standardisasi Nasional (BSN) applied a standard for shrimp paste products through Standar Nasional Indonesia (SNI) number 2716: 2016. Water content, protein content, and salt content are designated maximum at 45%, minimum at 15%, and between 12-20%, respectively. The result of proximate analysis shown that the nutritional level were out of standard. Changes in microorganism community and no standard in raw materials and procedures such as drying process and different salt addition might contribute to these results.

The most intriguing result from this study was physiological activity performed during fermentation, where metabolic shifting occurred between polymer and amino acid to phenolic utilization. In addition, nutritional content also affected by which microorganism performed different physiological activity. This study has successfully described the physiological function of the microorganism community during fermentation and its effect on nutritional content, thus can be used as the basis for shrimp paste quality improvement.

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