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A Comparison between Ice and Salt Storages on Bacteriological Quality of Asian Seabass (*Lates calcarifer*)

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Abstract: The study compares the bacteriological quality on Asian seabass (*Lates calcarifer*) between ice and salt storage methods. The main objectives of the study were to identify different bacteria constituents and quantitative bacterial load in Asian seabass when preserved with ice and sea salt. For the purpose of this study, Asian seabass was stored in two different conditions of ice-chilled and salted for 2 days. All fish samples were analyzed by performing bacteriological analysis and the isolated bacteria were identified by using API® identification system. In case of the quantity of bacteria in the flesh, Chilling and salting had no significant difference to the quantity of bacteria on fish flesh. As for the skin, salt-preserved fish showed higher quantity of bacteria than ice-preserved fish. *Acinetobacter baumannii* and *Pseudomonas fluorescens* had been identified from skin sample of ice-chilled fish. Besides *P. fluorescens* and *A. baumannii* other isolates identified include *Vibrio* and *Myxobacteria*. All bacteria were cocci-shaped except a few bacilli. In term of bacteria number and morphological characteristics, ice-chilled preserved fish was better than salt preserved fish. Overall, less number of bacteria was observed in both ice-chilled and sea salt preserved fish. The result of this study indicated that the quick preservation is a very important factor to control bacterial load in the preserved fish.

Key words: *Lates calcarifer*, ice storage, salt storage, bacteria, cocci-shaped

INTRODUCTION

Fish contributes about 60% of the world's protein supply and 60% of the developing world derives more than 30% of their annual protein from fish (FAO, 2007; Kumolu-Johnson and Ndimele, 2011). In Malaysia, fish is an important part of human diet as it is one of the major cheap protein sources. Malaysia is a country that surrounded by the sea and fisheries is one of the country's major industry. Among various fishes, Asian seabass (*Lates calcarifer*) is one of the most popular fish in Malaysia. It is an economically important food fish in Malaysia. Malaysian people prefer Asian seabass due to its delicately flavoured flesh. However, total production of Asian seabass in Malaysia is increasing day by day. In 2004, in Malaysia, total production of Asian seabass was 5.7 thousand MT while in 2009 it was nearly 16 thousand MT (FAO, 2011). In Malaysia, the production of Asian seabass is mainly from aquaculture farms in east Malaysia, where Asian seabass is generally sold to the wet market and are also distributed to the supermarket.

Fish is a very perishable product therefore, the quality and freshness of fish decline rapidly upon dying. Microbial activity starts in the different organs of fish after dead and the quality of fish is gradually deteriorating. However, the rate of deterioration or bacterial activity depends directly on storage and processing conditions of fish. A good storage condition can stop microbial activity and spoilage. Storage conditions may also influence the sensory profile (Aaraas *et al*., 2004). Therefore, storage conditions play a very important role in maintaining the quality of fish. To maintain the quality, fish and fishery products are stored or preserved for years using a wide range of technological processes. These include curing, freezing, chilling, canning, etc. Curing method includes salting drying, smooking and pickling. However, in many cases, applied preservation technology is greatly dependent on types of

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fish. Selection of proper storage and preservation methods are also based on duration of storage and the quantity of fish to be stored (Burt *et al*., 1992).

In practice, Asian seabass is generally kept in chilled condition using crushed ice for a short-term storage. However, chilling does not kill the microorganisms but reduces microbial metabolism which is responsible for spoilage. Other alternative might be using sea salt (sodium chloride). Sea salt has been using for centuries as a preservative agent for many fishes (Takiguchi, 1989). Salting lengthens the shelf life of fish and fishery products. Besides preservation, it also acts as enhancer attractive flavor of many fishes. There are many studies which discuss the bacteriological quality at different storage conditions of different fishes (Aaraas *et al*., 2004; Tejada *et al*., 2007; Zambuchini *et al*., 2008). Unfortunately, there is no study that discuss about bacteriological quality of Asian seabass when stored with crushed ice and sea salt. Therefore, the proposed study was conducted to evaluate the bacteriological quality of Asian seabass when preserve with crushed ice and sea salt. The main objectives of the study were to identify different bacteria in Asian seabass when preserve with ice and sea salt, and to compare their quantitative bacterial load.

MATERIALS AND METHODS

Sample collection and storage: A total of six live seabass was collected from a marine fish cage at Kuala Pahang and transferred to the laboratory and allowed to die in container with ice. All fishes were mixed and divided into two groups based on random selection. Each group contained three fish, each of which were considered as a replication in this study. The first group was stored into a plastic container with sea salt while the second group was stored with crushed ice. Both of the storage conditions were maintained for two days.

Examination of morphological characteristics of fish: The basic morphological characteristics of fish were examined in the laboratory before and after storage conditions. The basic morphological test of fish included appearance of skin, eyes and gill. Skin was examined for their firmness and shiny characteristic while cornea of eyes was examined for its clarity and the red colour criteria for the gills.

Microbiological analysis: Microbiological analysis was done according to Buller (2004). It involved the isolation and inoculation of the bacteria from selected parts of the fish samples followed by subsequent incubation and subcultures. Nutrient agar was used specifically as the general media and nutrient broth was used as the media for subculturing pure bacteria colonies.

The fish samples were swabbed using sterile cotton tips at two different parts: skin and flesh. A special care was taken to maintain an equal swabbing at each time. Then, the swabs were directly inoculated into nutrient agar plates. For the skin, swabbing was made at the surface of each of the fish using sterile cotton tips. In order to swab the flesh part, a fillet cut was made at the posterior part of the fish using a sterile blade. Then swabbing was made using sterile cotton tips. Direct inoculation to the nutrient agar plate was made upon the cotton tip was swabbed on the appropriated part by a single streaking then followed by several subsequent streaks over the first streak using sterile inoculating loop.

The primary isolation plate media were incubated at 30°C for 24 h. After incubation, each culture plate was examined with the dissecting microscope for any appearance of discrete bacterial colony growth. To obtain pure growth, a representative of each colonial type has been picked and subcultured to nutrient agar plates which served as secondary plates. In this step, the primary culture of plate was examined and identified how many colonies had similar size, morphology, color, wrinkled, mucoid, spreading, clear, etc. and then was selected, circle and number them with a marker or wax pencil. After that, colonies were picked to be isolated. The subculturing of single colonies was performed by using dilution-streaking technique and then was incubated at 30°C for 24 h. After that, each pure colony was inoculated into Falcon tube containing 30 mL nutrient broth and further incubated at 30°C for 24 h. This was served as a stock culture for each pure bacterial colony.

Gram-positive bacteria and Gram-negative bacteria were identified using Gram staining which was done based on American Society of Clinical Pathology (ASCP, 2004) method with some modification. Firstly a bacterial smear was prepared from a freshly cultured bacterial colony (18-24 h old). Then, a drop of water was placed onto the glass slide. A minute amount of a bacterial colony was spread evenly after aseptically transferred onto the water drop and allowed to fully dry. The procedure was continued with the fixation of the specimen onto the glass slide by gently passed the underside of the slide through a flame 2 to 3 times and the slide were let cool.

The smear was flooded with crystal violet solution and kept for 30 sec before being rinsed with water. After that, the slide was flooded again with iodine for 30 and 60 sec before being rinsed with water. An iodine solution was used as a mordant. A mixture of acetone and alcohol was added to the slanted slide drop-wise until no colour appears in the drippings or for about 5 seconds. Then, the slide was rinsed with water immediately. Lastly, the slide was flooded with safranin for 30 to 60 sec before being rinse gently with water until no colour appear in the effluent. The preparation of smear was completed by

blotting the smear dry or letting the smear air-dried. After that it was examined under the light microscope to observe the cell shape and differentiate whether it is gram negative or positive bacteria.

Identification of fish pathogenic bacteria was done using API® 20E test, which uniquely used for identifying Enterobacteriaceae and other non-fastidious Gram-negative rod. The API® 20E kit is not for direct use with the specimens thus the bacterial samples were allowed to grow on fresh nutrient agar plates first and then incubated for 18 to 24 h at 30°C prior to use with the kit. API® test uses 21 standardized and miniature biochemical tests. The reactions were read according to the table provided with the kit. For this, an incubation box that consisted of a tray and a lid was prepared. Then, a humid condition was formed in the honey-combed wells of the tray by distributed about 5 mL of distilled water. The reference of the bacterial isolate was written on the elongated flap at the end of the tray for recording purpose. After that, the strip that contains 21 micro- tubes was placed on the tray.

A single well-isolated colony from nutrient agar plate was taken and transferred into a universal bottle containing 5 mL of 0.85% sterile normal saline solution. Different bottle containing sterile normal saline solution was use for each different bacterial isolates. Then, the solution was homogenized and distributed into the 21 microtubes using micropipette. For CIT, VP and GEL tubes were filled with suspension into both tubes and cupules. Then, ADH, LDC, ODC, H_2S and URE tubes were overlaid with sterile mineral oil, filling the cupules to create anaerobic atmosphere. After that, the strip was put into the incubation box and incubated at 37°C for 18 to 24 h. After the incubation, the results were read by the Reading Table in the manual of the API® 20E kit. In the case where the number of positive test (including the GLU test) was less than 3, the strip was reincubated for a further 24 h without any addition of any reagent. The strip was analyzed after the incubation if more than three tests were positive (including GLU test). If not, the strip was further incubated for another 24 h without adding any of reagents.

For TDA test, 1 drop of TDA reagent was added into the TDA tube. A positive reaction was indicated by a reddish brown color while negative reaction indicated by yellow color. For IND test, 1 drop of JAMES reagent was dropped into the IND tube. A positive result was shown by a development of a pink color. A negative result was indicated by yellow color. IND test was performed last because gaseous product was released in the reaction which could interfere other tests interpretation. For VP test, 1 drop of each VP 1 and VP 2 reagents were dropped into VP tube and in 10 minutes, positive reaction occurred with a pink or red color was observed. In contrast, if there was formation of slightly pinkish color after 10 min, the test was considered negative. All the results were recorded on the result sheet and analyzed by using the api*web*™ identification system.

Several supplementary tests (nitrate reduction test, motility test, growth on MacConkey agar, oxidation of glucose and fermentation of glucose) were carried out to confirm the characteristic and identification of the bacteria. Nitrate Reduction Test was performed after completely adding reagent to TDA, VP and IND microtubes after incubation of API 20E strip. 1 drop each of NIT 1 and NIT 2 reagent were added to the GLU microtube of the API 20E strip. After 2-5 min, a red colour indicated a positive reaction (reduction of nitrate to nitrite) while yellow colour indicated a negative reaction. When negative reaction happened, 2-3 mg of zinc dust was added to the GLU microtube. If the tubes remained yellow after 5 min, it indicated a positive reaction to be recorded on the results sheet. If the test turned orange-red, it was a negative reaction.

Motility Test was performed on SIM medium. An inoculating needle was used in this test to pick a colony from a fresh bacterial culture. The inoculating needle was then stabbed into the centre of SIM medium contained in a capped bottle. This bottle was incubated for 24 h at 30°C. Positive reaction was indicated by fuzzy streak, fan shape pattern, nodular growth of the bacteria and also turbidity in the medium while negative reaction was indicated by accentuated bacterial growth along the stab line. For the test of bacterial growth on MacConkey agar, fresh inoculums were streaked on MacConkey agar plate by using an inoculating loop. This was followed by incubation at 30°C at 24 h. Presence of bacterial growth on the agar was considered as positive reaction while the absence of bacterial growth was recorded as a negative reaction. OF basal medium was used to observe the activity of glucose oxidation by the bacteria. With an inoculating needle, a fresh single was picked and stabbed into the OF media contained in a capped bottle. Then, it was incubated at 30°C for 24 h. The same procedure was repeated to test the fermentation activity of bacteria. The different was, a layer of sterile mineral oil about 1.5 mL was added to the top of media after the colony as stabbed into the OF media. For both oxidation and fermentation reaction, positive reaction was indicated by yellow colouration while green colour indicated negative reaction.

RESULTS

The basic morphological condition of fish before and after storage conditions are presented in Table 1. Before storage, skins of all seabass were firm and shining, corneas of eyes were very clear and gills of all seabass were red colour. Asian seabass was not different after two days of storage with crushed ice except appearing some trans parent slime on the skin. However, some changes

Table 1: Morphological changes of Asian seabass after two days of ice and salt storage

Condition	Hours	Skin	Eyes	Gills
Ice-chilled storage	0	Firm, Shining,	Clear Cornea	Red
	48	Firm, Shining, Transparent slime	Clear Cornea	Red
Sea salt storage	0	Firm, Shining	Clear Cornea	-
	48	Coarse, Dried	Dried Clear Cornea	-

Table 2: Result of Gram Staining of bacteria sample

Isolate	Colour	Result	Shape
Sample	Purple	G+	Bacilli
CF 1	Purple	G+	Cocci
SF 2a	Purple	G+	Cocci
SF 2b	Purple	G+	Cocci
SF 3	Pink	G-	Cocci
CS 1a	Purple	G+	Cocci
CS 1b	Pink	G-	Cocci
CS 2	Pink	G-	Bacilli
CS 3	Purple	G+	Cocci
SS 1a	Purple	G+	Cocci
SS 1b	Purple	G+	Cocci
SS 1c	Purple	G+	Cocci
SS 2a	Purple	G+	Cocci
SS 2b	Purple	G+	Cocci
SS 2c	Purple	G+	Cocci
SS 3a	Purple	G+	Cocci

CF: Flesh of ice-Chilled storing fish, CS: Skin of ice-Chilled storing fish, SF: Flesh of sea salt storage fish; SS: Skin of sea salt storage fish, Numeric value under isolate column indicates replication number. a, b, and c indicate different isolate in the same plate. G+ and G-:Gram positive and Gram negative bacteria, respectively

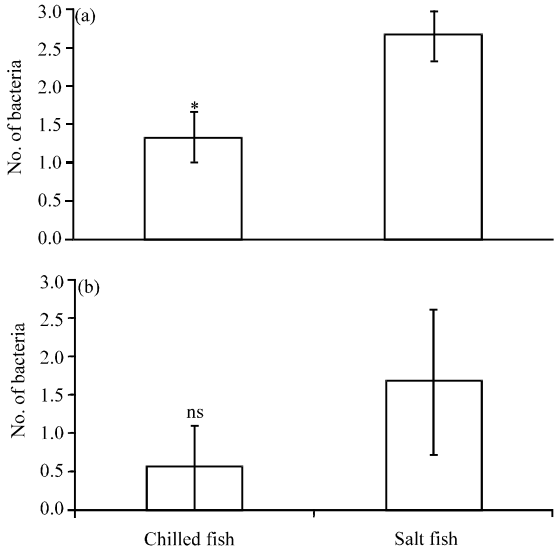

Fig. 1(a-b): Effect of chilling and salting on bacteria number in (a) Skin and (b) Flesh. A *Significantly different at $p<0.01$ and ns: Not significantly different, Data are Mean±SD

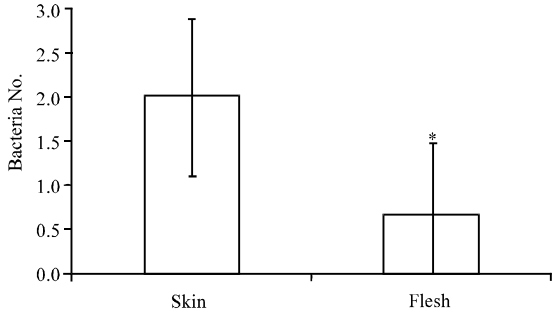

Fig. 2: Bacteria number in skin and flesh after two days of storing. *Significantly difference ($p<0.05$) between the bacteria number. in skin and flesh. Data are Mean±SD

were observed in sea salt storage seabass. After two days, skin of salt storage seabass was coarse and dried. Although their eyes were also dry but showed clear after storing two days with sea salt.

The effect of ice-chilling and salting on bacteria number in flesh and skin are shown in Fig. 1. In case of bacteria number on skin, sea salt storage fish was statistically different that chilled fish. Sea salt storage seabass had more bacteria than ice-chilled seabass. However, in case of bacteria number in flesh, there was no significant different ($p>0.05$) between ice-chilled and salt storage seabasses. There was a significant difference on the bacteria number in skin and flesh ($p<0.05$). Overall more bacteria were observed at the skin than in flesh (Fig. 2).

A total of 16 different isolates were distinguished in the present study. Out of 16 isolates, only 3 (19%) isolates were identified as gram negative while the rest 13 (81%) were gram positive (Table 2, Fig. 3). Among 16 isolates, only 2 isolates were identified as Bacilli and other 14 isolates were identified as cocci. API® test could only identified gram negative bacteria. In the present study API® test successfully identified 2 types of bacteria to its species level (Table 3). The bacteria were *Acinetobacter baumannii* and *Pseudomonas fluorescens*. However, both bacteria were identified in the skin sample ice-chilled, storage fish. The identified bacteria were classified into either fish spoilage bacteria or human pathogenic bacteria. However, *A. baumannii* was classified into human pathogenic bacteria while *P. fluorescens* was classified as fish spoilage bacteria. Besides *Pseudomonas fluorescens* and *Acinetobacter baumannii* many isolates were identified up to genus level. These included *Vibrio* and Myxobacteria.

Table 3: The most probable genus and species of isolates from Asian seabass identified by the API® identification system

Isolate	Possible bacteria
CS 1a	*Acinetobacter baumannii/calcoaceticus*
CS 2	*Acinetobacter baumannii/calcoaceticus*
CS 3	*Pseudomonas fluorescens/putida*

CS: Skin of ice-Chilled storing fish, [1,2]Replication No. [a]Different isolate in the same plate

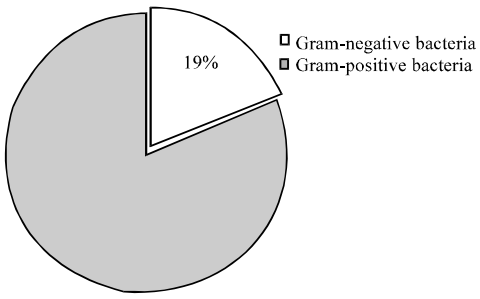

Fig. 3: Distribution of Gram-positive and Gram negative bacteria

DISCUSSION

This study focuses on bacteriological quality between sea salt and ice storages Asian sea bass to identify the better storage method. In the present study, result of morphological characteristics of fish indicated that all fish were fresh even after two days of storing. However, ice-chilled storage fish showed better morphological characteristics than that of sea salt storage fish. Before storage skin of Asian sea bass was firm, shining but after two days, skins of salt stored Asian seabass were coarse and dried. This indicated some chemical/bacteriological changes in the skin skins of salt storage seabass. There is no previous study comparing morphological characteristics between the ice and salt storage Asian seabass. However, this result concurs with the result of bacteriological study.

In the present study higher number of bacteria was observed on the skin of salt preserved fish than on the skin of ice preserved fish. Higher number of bacteria might cause more deterioration of the skin of sea salt preserved fish. Our result in a way agree with the result of Slabyj *et al.* (2003), who observed that chilled fishes samples had succeed preventing invasion and stopped bacteria from growing. The reason might be due to flash out effect of melted ice. In the case of ice preserved fish, water produced from melted ice removed bacteria from the skin. This reduced the number of bacteria on the skin of ice preserved fish. Another reason might be short time preservation. After 2 days, salted fish was not fully dried that bacteria might be survived in that condition. Therefore, storage time is another important factor for selecting appropriate storage method (Burt *et al.*, 1992).

In this study, comparatively higher quantity of bacteria was observed on the skin than in the flesh. This pattern of bacterial population distribution in fish body was also demonstrated by many previous researches (Huss *et al.*, 1995), Slabyj *et al.* (2003) who reported microbial growth mostly takes places at the surface of the fish after the deterioration the quality of fish while only a limited number of microorganisms actually invade in the fish flesh. According to Slabyj *et al.* (2003), this is probably due to a consequence of bacterial enzymes, which starts working in the flesh resulting in a lot nutrients diffuse out. This diffused nutrient rapidly accelerates bacterial growth on the skin of fish.

In the present study, overall a few bacteria (16) were observed in both salt and ice storage conditions. These indicate that the fishes were very fresh even after two days of storing. The reason of freshness might be storing immediately after dying. In this study, all fished were transported in the laboratory as live condition and they were stored immediately after dying by cold shock. Bacteria did not get enough time to increase their number before storing. Therefore, storage time (time between dying and storage) is a very important factor in maintaining quality of fish. Just after death, fish can be soft for a few hours (pre-rigor condition) but then it starts to become stiff. This phenomenon is called "rigor mortis". The fish stays in the "rigor mortis" condition for a while, but then its flesh muscles become relaxed again (NZIC, 2008). At this stage, the quality of fish starts to deteriorate very rapidly (Aaraas *et al.*, 2004).

Among all identified bacteria, most of the bacteria Gram positive. According to Huss *et al.* (1995), Gram negative bacteria is more dominant over gram positive bacteria in fish. However, this depends on many factors such as fish species, location of fish body from where sample is taken, storage time after dying, habitat of fish, etc. In this study, *A. baumannii* and *P. fluorescens* bacteria were identified from chilled fishes. This result in a way agrees with the result of Huss *et al.* (1995), who reported that *P. fluorescens* generally known as fish spoilage bacteria and observed in ice-chilled fish. However, according to Gerischer (2009), *A. baumannii* is a human pathogenic bacteria within the *Acinetobacter* genus or also known as opportunistic human pathogen. Besides *P. fluorescens* and *A. baumannii* many isolates were identified up to genus level. These included *Vibrio* and *Myxobacteria*.

CONCLUSION

In term of bacteria number and morphological characteristics, Ice-chilled preserved fish was better than

salt preserved fish. More research is needed to compare between salt and ice storages when fish are stored for a long period. Overall, less number of bacteria was observed in both Ice-chilled and sea salt preserved fish. The result of the present study indicated that the quick preservation is a very important factor to control bacterial load in the preserved fish. However, this research cannot give any conclusion in the case of long term preservation. Therefore, more research is recommended on the effects of chilling and salting on bacteriological quality if fish is preserved for a long term basis.

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