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To cite this article: F Ariyani et al 2018 IOP Conf. Ser.: Earth Environ. Sci. 139 012041

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The use of preservatives consist of green tea, piper betel and potassium sorbate on boiled salted fish processing

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Abstract. The main problem in boiled salted fish ikan pindang is mucus and mold on the surface of the fish which is produced relatively fast as well as the high level of histamine content especially when scombroid fish species are used as raw material. This study was performed to evaluate the effectiveness of various preservatives to overcome such problems. Three combinations of preservatives P1 (green tea and sorbate), P3 (green tea, piper betel, sorbate), P4 (green tea and piper betel) and P0 (no preservative/control) resulted from the previous study were used in this study. Before being used, the preservatives were tested against deteriorating microorganisms commonly found in boiled salted products, of which the result showed that all microorganisms were inhibited. The preservatives were then applied at three different stages of the process of boiled salted fish, i.e. before boiling, during boiling and after boiling. Sensory attributes and microbial characteristics of the products were then evaluated. The results showed that the performance of all tested preservatives against deteriorating microorganisms was relatively similar. It was also shown that the application before and during boiling performed better.

1. Introduction

Boiled salted fish ikan pindang is one of the traditional fisheries products which is popular in Indonesia as well as in ASEAN Countries. In the Philippines, this product is named siangeng, while in Thailand is named pla-tu-neng [1]. There are three kinds of boiled salted fish products available in the market, i.e. pindang naya, pindang badeng and pindang presto. The first two are the original pindang products of which the process involving heating the fish (usually scombroid fish) in solar salt (salt made from evaporation of seawater or other brine in the sun) and brine solution respectively. While pindang presto is a modified traditional process involving high-pressure cooking. Milkfish is commonly used as raw material and the product is vacuum packed.

In pindang naya processing, the fish is arranged in a bamboo basket and then boiled in 15 % brine solution for approximately 30 min. After being cooled, the product is transported and sold in the market. As the salt content is low, while the packing is not perfect, this product has a short shelf life (1–2 days). Bacterial and mould contamination during transportation and marketing contributed to the short shelf-life of the product resulting in slimy and mouldy products, stale taste and odor and even made the consumer feel itchy when scombroid fish is used.

Different methods of preservation have been used to extend the shelf-life of boiled salted fish such as low-temperature storage and proper packaging. Several natural products have also been studied to preserve boiled salted fish, such as guava leaf [2], chitosan [3], lemon grass [4] and green tea leave [5], all of which was applied as a single preservative.
Betel (Piper betle L) leave extract was reported as a potential antioxidant, antibacterial as well as antifungal agent [6, 7, 8, 9, 10, 11]. Betel leave extract was able to reduce histamine producing bacteria of fresh mackerel (Rastrelliger kanagurta) kept at ambient temperature [12]. Meanwhile, potassium sorbate was reported to be able inhibiting mold and yeast [13, 14] while sorbic acid could inhibit the growth of mold on smoked milkfish during refrigerated storage [15], and on fishloss [16].

All of the above studies used the preservatives as a single preservative. The existing study reports the used of multiple preservatives, i.e. green tea leave, betel leave and potassium sorbate to produce a better quality of pindang naya.

2. Materials and Methods

2.1. Materials

Commercial green tea was purchased from tea processing plant, Gambung, West Java. Betel leave was collected from Biofarmaka IPB and potassium sorbate was purchased from PT. Setia Makmur Jakarta.

The bacteria used in this study were M. morganii ATCC® 25830™, K. pneumonia ATCC®13883™, P. fluorescens ATCC® 13525™, P. aeruginosa ATCC® 10145™, A. flavus ATCC® 26949™ and P. citrinum ATCC® 9849™ collected from Food and Nutrition Culture Collection of IUC Food and Nutrition, Universitas Gadjah Mada, Yogyakarta.

Fish used as raw material of dried salted fish was skipjack (Euthynnus affinis) with the size of 22–25 cm long and 126–163 g weight, collected from a fish collector in Muara Baru, Jakarta.

2.2. Preparation of preservatives

Water extract of leaves was prepared according to Arambewela et al. [17]. Fresh leaves were washed to remove dust, debris and other foreign material, drained and dried at room temperature (27–32 °C). Dried leaves were then boiled in water with the proportion of dried leaves and water was 1:4 (w/v) for 4 h, cooled at ambient temperature and filtered using nylon fabrics of 30 mesh. The filtrate was packed in a dark plastic container and kept frozen until being used.

Preservatives were prepared by the formulation of green tea extract, betel leave extract and potassium sorbate to set three formulae, i.e. P1 (consists of green tea and sorbate), P3 (consists of green tea, piper betel, sorbate) and P4 (consists of green tea and piper betel).

2.3. Preparation of bacterial suspension

Each stock culture of bacteria (M. morganii, K. pneumonia, P. fluorescens and P. aeruginosa) were cultured on nutrient broth at 35 °C for overnight. After incubation, the culture was streaked on nutrient agar (slant agar), incubated at 25 °C for 3–5 days, and then stored in the refrigerator. The bacteria were then placed into a test tube filled with 10–15 mL nutrient broth, incubated for 24 h and then set the concentration of the inoculum to 0.5 McFarland’s standards (ca. 10^6 cfu·mL^-1).

2.4. Preparation of fungi suspension

Each stock of fungi (A. flavus and P. citrinum) were cultured on Potato Dextrose Broth (PDB) media, incubated at 25 °C for 3–5 days. After incubation, the culture was streaked on Potato Dextrose Agar (slant agar), incubated at 25 °C for 3–5 days, and then stored in the refrigerator. The fungi were placed into a test tube filled with PDB, incubated for 48 h and then set the concentration of the inoculum to 0.5 McFarland’s standards (ca. 10^6 cfu·mL^-1).

2.5. Antibacterial and antifungal assay

Antibacterial activity of the preservatives was tested against spoilage bacteria P. aeruginosa ATCC® 10145™ and P. fluorescens ATCC® 13525™ and against histamine forming bacteria M. morganii ATCC® 25830™ and K. petricola ATCC® 13883™, while antifungal activity was tested against A. flavus ATCC® 26949™ and P. citrinum ATCC® 9849™. Both antibacterial and antifungal activities were measured using disc-diffusion method [18]. Sterile paper discs (6-mm diameter) were soaked in
the preservatives for 15 min and allowed to dry for 10 min. The inoculum suspension of 0.15 mL was swabbed uniformly on the surface of Mueller Hinton agar plates, allowed to stand 15 min and the disk was placed in different areas on the plate. They were incubated at 37 °C for 24 h for the test bacteria and at room temperature for 2–4 days for fungal strains. The blank disc was used as negative control, while antibiotic of chloramphenicol (30 µg) was used as positive control. The plates were duplicated in all the experiments.

2.6. Application of preservatives on boiled salted fish processing

Skipjack as a raw material of boiled salted fish was washed to remove debris and blood. The processing of boiled salted fish consisted of boiling of fish in a brine solution (with a salt concentration of 15 %) for 30 min, draining and storing at ambient temperature.

The application of the preservatives (P1, P3 and P4) on boiled salted fish processing in this study was conducted at three different stages of the process, as a) soaking solution for fish before boiling process, b) boiling solution during boiling process and c) soaking solution after boiling process. Duration of soaking was 30 min. The process of boiled salted fish without adding preservatives was used as a control.

Samples were evaluated for sensory attributes, including color and taste [19] on the day of processing, while microbial characteristics including histamine forming bacteria [20], TPC [21] and total fungi [22] were observed on the day of processing and after being stored for 3 days. The treatment experiment was conducted in three replicates.

3. Results and Discussion

3.1. Antimicrobial activity

In this present study, evaluation of antibacterial activity of various preservatives was conducted against spoilage bacteria, histamine-forming bacteria and fungi.

Antimicrobial activity of preservatives against spoilage bacteria and histamine forming bacteria was presented in figure 1 and figure 2 respectively, while that against mould was shown in figure 3.

![Figure 1](image-url)  
*Figure 1. Inhibition zones of various preservatives on isolates of spoilage bacteria. (P0: no preservative/negative control; P1: green tea, sorbate; P3: green tea, piper betel, sorbate; P4: piper betel, sorbate).*

Figure 1 shows that all preservatives were effective against spoilage bacteria *P. aeruginosa* and *P. fluorescens*, and there was no significant difference among preservatives in term of their effectiveness against these bacteria, although the effect was still lower than that of antibiotics chloramphenicol. It seems that *P. aeruginosa* was slightly more sensitive than *P. fluorescens*. Combination of green tea,
betel leaves and potassium sorbate performed similar effectiveness to a combination of green tea and betel leave as well as green tea and potassium sorbate. Each component of preservatives has antibacterial activity against specific bacteria. Aqueous extract of betel leave has performed inhibition activity against *P. aeruginosa* with inhibition zones of 6 mm [23], while inhibition zone of 8 mm against the same bacteria was shown by 10 % betel leave extract [24]. In related to green tea extract, 10 % of aqueous green tea extract could inhibit *P. aeruginosa* with diameter inhibition zone of 12 mm and 9.4 mm, respectively [18, 24].

![Figure 2](image)

**Figure 2.** Inhibition zones of various preservatives on isolates of histamine forming bacteria. (P0: no preservative/negative control; P1: green tea, sorbate; P3: green tea, piper betel, sorbate; P4: piper betel, sorbate).

Related to histamine forming bacteria, the result of an experiment on disc-diffusion method indicated that all preservatives demonstrated zone of inhibition of 8.9–9.2 mm towards *M. morganii* and 7.8–8.5 mm against *K. pneumonia* (figure 2), however, their performance was still lower than antibiotics. It was shown in this study that *M. morganii* was more sensitive to tested preservatives than *K. pneumonia*. This supports the previous finding that green tea extract, one of the bioactive components in the preservatives was effective in inhibiting *Klebsiella pneumonia* [25]. Regarding betel leave, as another bioactive component present in this preservative, it was reported that nonaqueous extracts, namely ethanolic extract, petroleum extract and chloroform extract of betel leave were very effective in inhibiting *K. pneumonia* with a zone of inhibition of 23–33 mm [26]. Aqueous extract of green tea more effective toward *M. morganii* than *Klebsiella* spp., while betel leave showed comparable effectiveness towards both *M. morganii* and *Klebsiella* spp. [24].
The performance of preservatives in inhibited fungi activity was shown in figure 3. *Aspergillus flavus* seems more susceptible than *P. citrinum* when treated with preservatives, on the other hand, potassium sorbate as control positive was more effective towards *P. citrinum* than that of *A. flavus*. The previous study showed that betel leave extract at 1% completely inhibited the growth of *A. flavus* [27]. Green tea extract showed higher inhibitory effect towards *A. flavus* compared to *P. citrinum* [24], on the other hand, betel leave extract was more effective in inhibiting *P. citrinum* compared to *A. flavus*. Considering that *A. flavus* was more susceptible compared to *P. citrinum* in this study, it seems that the dominant effect of inhibiting microorganisms in this study was performed by green tea extracts.

3.2. Application of preservatives on boiled salted fish processing

3.2.1. Sensory characteristics. The results of the sensory assessment of boiled salted fish treated with various preservatives were shown in figure 4. The use of preservatives as a soaking solution before boiling as well as a soaking solution after boiling resulted in better color compared to the use of preservatives as a boiling solution. Preservatives used as boiling solution produced the darker color of boiled salted fish due to phenolic compounds present in green tea including catechin and its derivates [28] and also in betel leave such as chavicol, allylprotocatechol, chavibetol and hydroxychavicol [29]. During boiling process, these phenolic compounds are oxidized and further condensed to form components with a higher molecular weight having brown color [30].
Figure 4. Sensory characteristics of boiled salted fish treated with preservatives. (P0: no preservative/negative control; P1: green tea, sorbate; P3: green tea, piper betel, sorbate; P4: piper betel, sorbate).

The taste score of boiled salted fish treated with preservatives was not much different from that of negative control/without preservatives (figure 4). Panelists rate the taste of boiled salted fish in the range of 3.6–4.6 with a description of not bitter/not pungent–slightly bitter/slightly pungent. This indicates that addition of preservatives consisted of betel leave and green tea did not much effect on the taste of boiled salted fish. This could be due to the low concentration of preservatives used. It is known that sharp burning, pungent and astringent is the characteristics of betel leave taste [31], whereas green tea contained tannin have a bitter taste.

3.2.2. Microbiological characteristics

Figure 5. Total histamine forming bacteria count of boiled salted fish treated with preservatives. (P0: no preservative/negative control; P1: green tea, sorbate; P3: green tea, piper betel, sorbate; P4: piper betel, sorbate).

Based on microbiological analysis of boiled salted fish treated with preservatives, it was shown that soon after processing, total histamine forming bacteria slightly lower in boiled salted fish treated with
preservatives compared to that of control (figure 5). After being stored, the histamine-forming bacteria of treated boiled salted fish was significantly lower compared to control, except for the use of preservatives as a soaking solution after boiling process. The role of green tea contained catechin might be responsible for lowering histamine-forming bacteria, as informed by a previous study [5].

Treatment of boiled salted fish with preservatives slightly decreased total bacterial count especially the use of preservatives as a soaking solution before boiling process (figure 6). However, after 3 days of storage, it was less effective since total bacterial count increased significantly reaching up more than 5 log cycle. Boiled salted fish produced by boiling in a brine solution for 30 min (pindang naya) could be kept only for 2 days [32].

![Graph](image_url)

**Figure 6.** Total Plate Count of boiled salted fish treated with preservatives on a different step of processing. (P0: no preservative/negative control; P1: green tea, sorbate; P3: green tea, piper betel, sorbate; P4: piper betel, sorbate).

![Graph](image_url)

**Figure 7.** Total fungi of boiled salted fish treated with preservatives on a different step of processing. (P0: no preservative/negative control; P1: green tea, sorbate; P3: green tea, piper betel, sorbate; P4: piper betel, sorbate).
The main active component for antifungal effects is potassium sorbate. It is shown that boiled salted fish treated with preservatives (P1 and P3) contained lower total fungi although the products were stored for 3 days. It appears that the main active component for antifungal effects is potassium sorbate presence in preservatives P1 and P3. Potassium sorbate could inhibit fungi and yeast [13, 14]. This is in agreement with previous researchers [15], who prevented the growth of fungi on smoked milkfish by the use of sorbic acid.

4. Conclusion
In term of antibacterial and antifungal effects, the performance of all tested preservatives is relatively similar.

The use of the preservatives in boiled salted processing is suggested to be applied as a soaking solution before boiling or as a boiling solution.

Preservatives used as soaking before boiling produced boiled salted fish with slightly yellowish grey in color, no bitter taste and the lowest increase of both total histamine-forming bacteria (2.2 log number) and total fungi count (3.8 log number) after 3 days of storage.

Preservatives used as boiling solution was also suggested with boiled salted fish characteristics of brownish grey in color, no bitter taste and having an increment of total histamine-forming bacteria count of 2.9 log number and total fungi count of 4.0 log number after 3 days of storage.

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