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Phenotypic characterization and identification of bacterial isolates from smoked fish

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ABSTRACT

This study aimed to phenotypically characterize and identify bacterial isolates from smoked fish. From the seven morphologically different bacterial colonies, four of them were identified using selective/differential media. The two isolates belong to genus *Pseudomonas* while the remaining two were confirmed under genus *Staphylococcus*. Results of gram-staining, motility and selected biochemical and physiological tests supported the identity of the four isolates. *Pseudomonas* isolates were found resistant to 30 µg tetracycline while *Staphylococcus* isolates were intermediate to susceptible on the said antibiotics. Unhygienic preparation of the smoked fish could be the possible reason for the product contamination.

Keywords: Smoked fish, phenotypic characterization, biochemical test, physiological test, antibiotic susceptibility

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INTRODUCTION

The world population is growing drastically and thus, the demand for food has been increasing likewise. In the modern era, the awareness on the benefits of consuming nutritious food has made the demand for certain nutritious food stuffs, such as fish, to rank at the top of the highly demanded food stuffs. Fish and fisheries products are the most important nutritious food all over the world which represents about 15 to 20% of all animal protein on a global basis[1]. Fish is also a good source of omega-3 polyunsaturated fatty acids (PUFAs), micronutrients like vitamin D and different minerals[2].

However, fish is also known for its high perishability nature. The rapid deterioration of quality of fish and other seafoods after their harvest is mainly due to the various mechanisms of spoilage that takes place. The spoilage mechanisms associated with such deteriorations are grouped into microbial metabolic activities, endogenous enzymatic activities and chemical oxidation of lipids, all of which shorten the shelf life of seafoods[3]. According to Gram and Huss (1996), the high composition of non–protein nitrogen compounds and low acidity (pH>6) of the flesh of seafoods are the major cause of their spoilage, as these condition favor the growth of spoilage microorganisms. These microbes in turn produce metabolites that affect the organoleptic properties of the products and render them undesirable attributes[4].

The purpose of fish preservation is to reach the fish or fisheries product to an ultimate consumer in good and usable condition. Different types of fish preservation methods such as chilling, icing, freezing, sun drying, smoking, salting, fermentation and canning have been followed mostly in all the regions of Philippines to reach the fish or fisheries product to an ultimate consumer in good and usable condition and prevent or reduce the post-harvest losses[5]. Smoked fish are well accepted food items in the country. Smoking is the method of fish preservation effected by a combination of drying and deposition of naturally produced chemicals resulting from the thermal breakdown of wood[6]. Smoking gives the product a desirable color, taste and odor, a longer shelf-life through its antibacterial and oxidative effect, lowering of pH and acts as antagonist to spoilage[1,8,9,10].

The nutrients present in fish provide a good medium for microbial growth. Spoilage and other disease-causing organisms are introduced during handling, processing, packaging and storage[11]. Smoked fish and shellfish products can be a source of microbial hazards including *Listeria monocytogenes*, *Salmonella* spp., *Clostridium botulinum* and coliform bacteria[12].

Objectives of the Study

This study aimed to phenotypically characterize and identify bacterial isolates from smoked fish. The characterization mainly focused on selected morpho-biochemical and physiological traits, and tetracycline susceptibility of the isolated bacteria. Selective media were used in the process of identification.

MATERIALS AND METHODS

Isolation of Bacterial Colonies

Smoked fish sold in the public market of Muñoz, Nueva Ecija, Philippines was macerated using disinfected mortar and pestle. Two series of 10-fold dilutions (10^{-1} and 10^{-2}) of the macerated smoked fish was made in Trypticase Soy Broth (TSB). One hundred microliters (100 µl) of the diluted sample was spread into Trypticase Soy Agar (TSA) plates. The plates were incubated at 37 ˚C for 18 to 24 hours.

Cultural Characterization of Bacterial Colonies

Bacterial colonies grown in MHA plates were meticulously sorted based on their appearances on the medium. Colony characters such as size, color, optical property, shape, margin, elevation and texture were considered. Colonies that exhibit different cultural characters were grown in selective media, and were subjected to gram-staining, selected biochemical and physiological tests, and tetracycline susceptibility test.

Presumptive Identification of the Isolated Colonies Using Selective/Differential Media

Three selective/differential media were used namely LS (Lactobacillus Streptococcus) Differential Agar for detection of *Lactobacillus* spp. and *Streptococcus* spp., Staphylococcus Selective Agar for detection of *Staphylococcus* spp. and GSP (Glutamate Starch Phenol) Agar for detection of *Pseudomonas* spp. and *Aeromonas* spp. The media were prepared following the manufacturer's instruction. The culturally characterized colonies were streaked in the prepared selective/differential media. The plates were incubated at room temperature for 24 to 48 hours. Bacterial growth was observed the following day.

Gram Staining

The isolate was streaked on TSA plate and incubated at 37 ˚C for 18 to 24 hours. A smear was prepared by mixing a small amount of growth with a drop of distilled water. The smear was air-dried and fixed by heat. The glass slide was labeled properly. The dried smear was stained with crystal violet for 1 minute and was rinsed thoroughly with tap water. Afterwards, the smear was covered with Gram's iodine for 1 to 2 minutes and was washed with tap water. The smear was decolorized by dripping 95% ethanol and was washed immediately. Then, the smear was counterstained with safranin for 45 seconds and was washed by tap water. The slide was examined under microscope. Gram-positive bacterium should be colored blue while Gram-negative bacterium should be colored red. Cell size, shape and arrangement were also noted.

Catalase Test

The isolate was streaked on TSA plate and was incubated at 30 ˚C for 18 to 24 hours. A loopful of the bacterium was transferred to a clean slide. One to two drops of freshly prepared 3% hydrogen peroxide (H_2O_2) were dropped into the slide. Bubble formation indicates presence of catalase enzyme.

Citric Acid Utilization Test

The isolate was inoculated to Simmon Citrate Agar (SCA) slant using a wire loop by stabbing the butt and streaking on the surface. The slant was incubated at 33 ˚C for 48 hours. Growth and shift of the green color to Prussian blue color means positive utilization of citrate.

Urea Hydrolysis

The isolate was inoculated heavily to Christensen's Medium (CM) Urea broth. The tube was incubated at 35 ˚C for 4 to 6 hours. A red to violet color means positive test for urea hydrolysis.

Phenylalanine Deamination Test

The isolate was inoculated to Phenylalanine Agar (PA) slant. The slant was incubated at 33 ˚C for 18 to 24 hours. Four to five drops of 10% ferric chloride solution was be added. The immediate appearance of an intense green color indicates positive phenylalanine deamination.

Hydrogen Sulfide (H_2S) Production and Motility

The isolate was inoculated to SIM medium slant by stabbing the butt and streaking the surface. The slant was incubated at 30 ˚C for 48 hours. Browning on the surface and along the line of puncture means formation of lead sulfide which is an indication of H_2S production from amino acids. Diffused growth from the line of inoculation indicates that the bacterium is motile.

Growth in 6.5% Sodium Chloride (NaCl)

The isolate was streaked in TSA plate supplemented with 6.5% NaCl. The plate was incubated at 30 ˚C for 24 hours. The plate was observed for the presence of bacterial growth.

Tetracycline Susceptibility Testing

About 2 to 3 colonies of the presumptively identified bacterium were suspended in TSB. The bacterial suspension was incubated for 1 to 2 hours at 37 °C and then adjusted to 0.5 McFarland turbidity standards. The adjusted suspension was streaked in TSA plate using a sterilized cotton swab. The antibiotics discs, tetracycline (30 µg), were placed on the surface of the inoculated plate using sterile forceps. The plates were incubated at 37 °C for 24 hrs. The zone of inhibition was measured using a ruler. The susceptible, intermediate and resistant categories of the isolates to tetracycline were assigned on the basis of the critical points recommended by the Clinical and Laboratory Standards Institute (CLSI).

Statistical Analysis

Statistical difference in zone of inhibitions was compared using One Way Analysis of Variance. Comparison of means was done using Tukey's Test.

RESULTS AND DISCUSSION

Cultural Characterization of Bacterial Colonies

Seven bacterial colonies were isolated from the 10^{-1} and 10^{-2} dilutions of macerated smoked fish sample bought in the market. The bacterial colonies were chosen based upon its colonial characteristics in TSA as shown in Table 1. Colony size ranged from pinpoint (D) to small (F and G) to medium (A, B, C and E). All colonies were white in color. Based upon optical property, majority of the isolates were shiny (A, B, C, E, F and G). The colony shape varied from round (B, C, D, F and G) to complex (A and E). Three of the isolated colonies had entire margin (D, F and G), some were wavy (B and C), curled (A) and lobate (E). Colony elevation ranged from

convex (A, D, F and G) to umbonate (B and C) to flat (E). Based on texture, isolates B, C and D were mucoid, isolates A and E were rough, and the remaining isolates were smooth (F and G).

Table 1. Colonial characteristics of the seven bacterial isolates from smoked fish.

Isolates	Size	Color	Optical Property	Shape	Margin	Elevation	Texture
A	Medium	White	Shiny	Complex	Curled	Convex	Rough
B	Medium	White	Shiny	Round	Wavy	Umbonate	Mucoid
C	Medium	White	Shiny	Round	Wavy	Umbonate	Mucoid
D	Pinpoint	White	Opaque	Round	Entire	Convex	Mucoid
E	Medium	White	Shiny	Complex	Lobate	Flat	Rough
F	Small	White	Shiny	Round	Entire	Convex	Smooth
G	Small	White	Shiny	Round	Entire	Convex	Smooth

Presumptive Identification of the Isolated Colonies Using Selective/Differential Media

All of the isolated colonies failed to grow in LS Differential Agar. Isolates F and G were confirmed as *Staphylococcus* spp. because of its luxurious and cream growth in Staphylococcus Selective Agar[13]. Meanwhile, isolates B and C were confirmed as *Pseudomonas* spp. because of its luxurious and red-violet growth in GSP agar as opposed to the yellow growth of *Aeromonas* spp. in the same agar (Table 2)[14].

Table 2. Growth confirmation of the isolates in three selective/differential media.

Isolates	LS Differential Agar	Staphylococcus Selective Agar	GSP
A	-	-	-
B	-	-	+
C	-	-	+
D	-	-	-
E	-	-	-
F	-	+	-
G	-	+	-

Gram Staining, Motility, and Selected Biochemical and Physiological Tests

To further elucidate the phenotypic characteristics of the presumptively identified isolates, Gram-staining, catalase test, and selected biochemical and physiological tests were performed (Table 3). *Pseudomonas* spp. isolates had rod cells, Gram-negative, motile and positive to catalase test and citric acid utilization, negative to urea hydrolysis, phenylalanine deamination and hydrogen sulfide production. One of the *Pseudomonas* spp. isolate could live in a medium supplemented with 6.5% NaCl. Meanwhile, *Staphylococcus* spp. had spherical clustered cells, Gram-positive, non-motile, positive to catalase test and citric acid utilization and negative to urea hydrolysis, phenylalanine deamination and hydrogen sulfide production. Both of the *Staphylococcus* spp. isolates could withstand 6.5% NaCl.

Pseudomonas spp. and *Staphylococcus* spp. possessed catalase enzyme that mediates the breakdown of hydrogen peroxide into oxygen and water. Therefore, the two bacteria had the ability to protect itself from the lethal effect of hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism. Both of them had the capability to consume citrate as carbon source. *Pseudomonas* spp. and *Staphylococcus* spp. lacked the enzymes amino acid oxidase, urease and cysteine desulfonase because of negative results on phenylalanine deamination test, urea hydrolysis and hydrogen sulfide production, respectively[15].

Table 3. Results on Gram-staining, motility and other biochemical and physiological tests.

Isolates	Gram Staining	Shape	Motility	Catalase	Citric Acid Utilization	Urea Hydrolysis	Phenylalanine Deamination	Hydrogen Sulfide Production	Growth in 6.5% NaCl
Pseudomonas 1	-	Rod	Motile	+	+	-	-	-	-
Pseudomonas 2	-	Rod	Motile	+	+	-	-	-	+
Staphylococcus 1	+	Spherical (cluster)	Non-motile	+	+	-	-	-	+
Staphylococcus 2	+	Spherical (cluster)	Non-motile	+	+	-	-	-	+

Tetracycline Susceptibility Testing

The two *Pseudomonas* spp. isolates were resistant to 30 µg tetracycline while *Staphylococcus* 1 and *Staphylococcus* 2 were categorized as susceptible and intermediate, respectively based upon the critical points recommended by the Clinical and Laboratory Standards Institute (CLSI). Highest and significant zone of inhibition was recorded in *Staphylococcus* 1 (Table 4). It is well known that most species of *Pseudomonas* showed significant degrees of intrinsic resistance to a wide variety of antimicrobial agents such as β-lactams, tetracyclines, chloramphenicol and fluoroquinolones. The main cause of the resistance of *Pseudomonas* spp. was due to the low non-specific permeability of its outer membrane to small, hydrophilic molecules[16,17]. Kelman et al. (2011) found out that

Staphylococcus isolated from ground meats showed 69% resistance to tetracycline[18]. In a separate study, *S. aureus* isolates revealed 56.7% resistance to tetracycline[19]. The result of this study was contradicting to the published reports of Kelman et al. (2011) and Akanbi et al. (2017)[18,19].

Table 4. Zone of inhibition and CLSI category of the presumptively identified isolates.

Isolates	Zone of Inhibition (mm) to 30 µg Tetracycline	CLSI Category
Pseudomonas 1	8.0±0.0^{c}	Resistant
Pseudomonas 2	8.0±0.0^{c}	Resistant
Staphylococcus 1	21.0±1.4^{a}	Susceptible
Staphylococcus 2	15.8±0.5^{b}	Intermediate

Different superscript was significant at p<0.05 Resistant = < 14 mm; Intermediate = 15 to 19 mm; Susceptible = > 20 mm

Occurrence of *Pseudomonas* spp. and *Staphylococcus* spp. in Smoked Fish

In the past, smoking was a form of food preservation because large amounts of salt and long smoking times were practiced. Today fish is smoked more for flavor and appearance, and the amounts of salt and smoke used are not sufficient to prevent bacterial spoilage. Unclean, insufficiently or inadequately cleaned processing equipment have been identified as a source of bacterial contamination[20]. During handling and preparation, bacteria are transferred from contaminated hands of food workers to food and subsequently to other surfaces[21]. Poor hygiene, particularly deficient or absence of hand washing has been identified as the causative mode of transmission[20]. Insects, birds and rodents have been recognized as important carries of pathogens and other microorganisms[22,23].

CONCLUSION

From the seven morphologically different bacterial colonies isolated from smoked fish, two of them belong to genus *Pseudomonas* and the other two to genus *Staphylococcus*. The bacteria were identified using selective/differential media. Results of Gram-staining, motility and selected biochemical and physiological tests supported the identity of the four isolates. *Pseudomonas* isolates were found resistant to 30 µg tetracycline while *Staphylococcus* isolates were susceptible and intermediate to the said antibiotics. Unhygienic preparation of the smoked fish could be the possible reason for the bacterial contamination.

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