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Effect of citrus lemon *(Citrus limon)* essential oil on microbial load of smoked clupeids (*Ethmalosa fimbriata* and *Sardinella maderensis*) during storage

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ABSTRACT: This study examined the microbial load of smoked clupeids (*Ethmalosa fimbriata* and *Sardinella maderensis*) during a 12-week storage. One hundred and sixty samples (160; 80 each of both fish) of freshly smoked *Ethmalosa fimbriata* and *Sardinella maderensis* were procured from Makun Omi, Ogun Waterside, Ogun State, Nigeria. The fish samples were divided into two parts and a part coated with the essential oils while the other part was not. The coated samples were put in white polyethylene bags and placed in cartons and the second part (uncoated) was packed in polyethylene bags and placed in cartons. These cartons were then stored at ambient temperature (±25°C) for twelve weeks. The samples were assessed weekly for spoilage organisms in a twelve-week shelf-life study (to know the types and number of microorganisms per sampling time). Preserved fish samples had significantly ($p<0.05$) lower microbial load (Bonga:9.50−2.20 x 10^6 CFU/g and Sardine:6.25−1.30 x 10^6 CFU/g) than unpreserved samples (Bonga: 12.50−26.25 x 10^6 CFU/g, Sardine: 10.65−25.35 x 10^6 CFU/g). Eight (8) bacteria (*Bacillus subtilis, Klebsiella aerogenes, Escherichia coli, Bacillus megaterium, Staphylococcus aureus, Enterobacter cloacae, Lactobacillus fermentum* and *Staphylococcus saprophyticus*) were isolated from both samples, with the unpreserved having pathogenic micro-organisms (*Klebsiella aerogenes, Staphylococcus aureus* and *Escherichia. coli*). Lemon essential oil is therefore recommended as a potent natural preservative for smoked clupeids. Packaging essential oil preserved smoked clupeids in polyethylene bags before being placed in a box is also recommended as a way of keeping out microorganisms that can spoil fish.

Keywords: Clupeids, essential oil, lemon, micro-organism, spoilage.

INTRODUCTION

Globally, fish is the most common source of protein for many homes (Olagbemide and Akharaiyi, 2019). The advantages of fish as food include its ease of digestion and high nutritional value. It is much sought after by a good percentage of the world's population, particularly in developing countries. Consumption of fish has been associated with improved cardiovascular health and other health conditions thereby constituting an important component of diet for many people (Ayeloja, 2016). The high protein and fat contents of fish though attract consumers is the reason for its quick spoilage as soon as it is harvested. Fish spoilage, therefore, occurs as a result of the action of enzymes and bacteria present in fish and also the chemical oxidation of fat which causes rancidity. Fish preservation involves the various methods used in the prevention of fish spoilage and lengthening of its shelf life.

These methods are designed to inhibit the activity of spoilage bacteria and fungi that result in the loss of fish quality (Eyo, 2001).

Smoking of fish from smoldering wood dates back to early civilization (Olokor *et al.,* 2007) and about 66% of preserved fish in Nigeria are smoked (Cheikyula and Awobode, 2014). Smoking is desirable partly due to the procedural ease and consumer preferences. Smoking as one of the methods for preserving fish is often adopted in most localities in Nigeria. Clupeids belong to the family *Clupeidae*; and include many of the most important food fishes (shads, herrings, sardines etc.) commonly caught for the production of fish oil and fish meal (Froese and Pauly, 2011a). Clupeids are the most valuable family food fishes in the world in the order *Clupeiformes* (Dewey, 2014). The flesh of clupeids is oily, a feature that adds greatly to their flavour and provides valuable oil for the fishery industry. Clupeids also have numerous intramuscular bones (Dewey, 2014) and are the main components of the Nigerian pelagic fishery which contributes about two-third of the total marine fishery resources (Dewey, 2014).

Ethmalosa fimbriata (Bonga shad (English) and *Agbodo* in the Yoruba language) belong to the family *Clupeidae* and order *Clupeiformes* (Froese and Pauly, 2011b). It is a coastal and estuarine clupeid found along the numerous estuaries of the Niger Delta Region. It is pelagic, anadromous and usually 0-50cm long (Plate 1). Bonga is the most important clupeid caught by seine net; and is a major source of animal protein for many Nigerians especially those living in the coastal areas and marketed in dried forms to all parts of the country (Idodo-Umeh, 2003). *Sardinella maderensis* is an oceanodromous pelagic filter-feeding clupeid. It is usually found in “schools” at either the surface or the middle of the water body (Idodo-Umeh, 2003). It is a silvery fish that is similar to the round Sardinella (*Sardinella aurita*) but differs with its grey caudal fins having black tips (Tous *et al.,* 2015) (Plate 2).

Citrus species are small to medium-sized shrubs or trees cultivated throughout the tropics and subtropics. Citrus is primarily valued for the fruit, which is either eaten alone (sweet orange, tangerine, grapefruit, etc.) as fresh fruit, processed into juice, or added to dishes and beverages (lemon, lime, etc.). All citrus species have traditional medicinal value (Darjazi, 2014). The lemon tree’s ellipsoidal yellow fruit is used for culinary and non-culinary purposes throughout the world, primarily for its juice which has both culinary and cleaning uses (Djenane, 2015). Lemons (Plate 3) are a rich source of vitamin C and also they contain numerous phytochemicals including polyphenols and terpenes (Rauf *et al*., 2014). The use of natural preservation methods has not gained wide application compared to the use of synthetic preservative methods. However synthetic preservation has been discovered to have a negative impact on human health. There is therefore need to intensify research on bio-preservatives and their efficacy in the preservation of fish in order to reduce post-harvest losses and health hazards posed by chemical preservatives (Toviho, 2014). The present study aims to isolate and identify the microorganisms responsible for the spoilage of stored smoked clupeids.

Plate 1. *E. fimbriata* (Bonga: 9) (Source: Field Survey, 2018).

Plate 2.*S. maderensis* (Sardine: 20) Source: Field Survey, 2018).

Plate 3. Lemon fruit (Source: Field Survey, 2018).

MATERIALS AND METHODS

Lemon (*Citrus limon*) fruits were bought from the Oje market in Ibadan, Oyo State and the identification was done at the Pure and Applied Botany Department of the Federal University of Agriculture, Abeokuta (FUNAAB) (Sneath and Sokal, 1973). One hundred and sixty samples (160; 80 each of both fish) of freshly smoked *Ethmalosa fimbriata* and *Sardinella maderensis* were procured from Makun Omi, Ogun Waterside, Ogun State, Nigeria. The fish was smoked using a mud kiln after the fresh samples were washed to remove dirt, brined and smoked with heat from smouldering coals till it was dried. The smoked fish were then spread on the shelf to cool before the storage experiment. The fish samples were divided into two and one part was coated with the lemon oil while the other part was not coated (Plates 4 to 7). The coated samples were packed in white polyethylene bags and placed in cartons and the uncoated part was packed in polyethylene bags. These samples were then stored at ambient temperature (±25°C) for twelve weeks. The samples were assessed for twelve weeks for spoilage organisms in a shelf-life study. This study was carried out to know the types and number of microorganisms associated with the smoked fish samples.

Enumeration and Isolation of microorganisms

Microorganisms were isolated from the skin, flesh and head part of the smoked fish. This was done wearing gloves, cutting and weighing the parts (10 g) and dissolved in 40 ml peptone water for serial dilution. The aliquots of 0.5 ml of serially diluted organisms were introduced into 3 Petri dishes each. Pure isolates were obtained by spreading bacteria on the surface of a molten Nutrient agar that has set so that a single cell occupies an isolated portion of the agar surface. This single cell was repeated to produce a visible colony of similar cells, or clones. Pure isolates were identified according to Ephraim's (2010) method of microorganism identification at the colonial and morphological levels.

Characteristics and identification of microorganisms

Pure cultures of the isolates were identified morphologically and biochemically according to Ephraim (2010).

Colonial characteristics

After appropriate incubation periods, a macroscopic examination of surface colonies on Nutrient Agar medium was done to determine the shape, pigmentation, edge, elevation, surface, arrangement and marginal characteristics of the isolates.

Plate 4. Uncoated Bonga (*E. fimbriata*) sample (Source: Field Survey, 2018).

Plate 5. Coated Bonga (*E. fimbriata*) sample (Source: Field Survey, 2018).

Plate 6. Uncoated Sardine (*S. maderensis*) (Source: Field Survey, 2018).

Plate 7. Coated Sardine (S.maderensis) (Source: Field Survey, 2018)\.

Gram staining

Smears of 24 hours old cultures of bacterial isolates on clean glass slides were heat-fixed and stained with crystal violet for 30-60 seconds. The dye was drained and then fixed with Lugol's Iodine for 30 seconds. The slides were rinsed under running tap water, decolourised with 95% ethanol for about 10-15 minutes and again washed under tap water. The smears were counter-stained with Safranin for 30 seconds, and then rinsed, air-dried and examined under the microscope using the oil immersion lens for Gram reaction and cellular morphology. Gram-positive microorganisms stained purple, while Gram-negative microorganisms appeared pink.

Spore staining

Certain spore-producing bacteria were detected using the spore-staining technique. Smears of a 48-hour-old culture of isolates were fixed using heat on different glass slides, then flooded with malachite green stain and heated over a beaker of boiling water for 10 minutes. More stain was added continuously to the slides to avoid drying. The slides were subsequently washed and examined under oil immersion lens. While the vegetative portion of the microorganism appeared pink or red, the spores stained green.

Biochemical tests for bacterial isolates

Motility test

The motility test was carried out on the isolates using the procedures of Ephraim (2010). A clean depression slide and cover glass were washed and rinsed to provide a grease-free slide. Then a very small amount of Vaseline was placed near each corner of the cover slide. Two loopful of the isolate were placed in contact with the cover glass with the depression slide put over the drop of suspended bacteria. The slide was quickly inverted and examined under the light microscope. Flagellated microorganisms were observed in constant motion.

Coagulase test

A generous loopful of the isolate was added to a tube of citrated plasma and thoroughly homogenized with the loop. The homogenized inoculums were incubated at 37°C for 1-4 hours. The incubated tube was examined at 30 minutes to hourly intervals for the first couple of hours for the presence of a clot by tipping the tube gently on its side. The coagulase positive test was indicated by a degree of clotting within 24 hours (Clinical Microbiology Procedures Handbook, 2016).

Catalase test

The principle of this test is that most aerobic microorganisms are capable of producing the enzyme catalase (although to different extents). A suspension of the 18-hour-old culture of the test organism was made with sterile distilled water on a clean glass microscope slide. A few drops of hydrogen peroxide (H_2O_2) were added using a dropping pipette. The evolution of gas bubbles caused by the liberation of free oxygen indicated the presence of catalase enzyme. On the other hand, the absence of catalase enzyme was indicated by lack of gas bubbles.

Oxidase test

This was carried out for the detection of cytochrome oxidase in the microorganisms. Bacti-dent oxidase test trips were inserted into overnight broth culture (18 to 24 hours). The strips were withdrawn at once and left for 10 minutes for colour change. Oxidase-positive microorganisms changed to dark purple within 5 to 10 seconds. Delayed oxidase-positive microorganisms changed to purple within 60 to 90 seconds. Oxidase-negative microorganisms did not change in colour (Shields and Cathcart, 2010).

Indole test

A loopful of the bacterial isolate was dissolved in peptone water in a test tube and incubated at 37°C for 24 hours. The Kovac test was performed for indole production by adding 8 drops of Kovac's reagent to the test tube using a glass dropper. The tube was allowed to stand for about 1 minute. Observation of a "Cherry red" colour indicated a positive indole production test while a golden yellow colour indicated a negative indole production test (Dahal, 2023)

Methyl Red-Voges Proskauer (MR-VP) test

The isolates were singly inoculated into 10 ml of MR-VP medium in a test tube or McCartney bottles and incubated at 35°C for 3 days. After incubation, the tests were performed as follows: MRTest: Five drops of Methyl Red indicator were added to the culture. A red colour indicated a positive reaction. VP Test: Five millilitres (5 ml) of Alpha-naphthylamine (APHA) reagent (mixture of 1g of copper sulphate dissolved in 40 ml of saturated sodium hydroxide solution plus 960 ml of 10% Potassium hydroxide solution) was added to the culture. A pink to red colour indicates a positive reaction while a yellow colour shows a negative (McDevitt, 2009). The colour change shows high acid production and a decrease in the pH of the culture medium.

Carbohydrate fermentation

The test microorganism was inoculated in a tube containing the carbohydrate medium (glucose), with an inverted Durham's tube and incubated overnight at 37°C. The change of colour from straw yellow to pink indicated fermentation of sugar and gas production was indicated by trapped air in the Durham's tube (Reiner, 2012)

Citrate utilization test

This is used to test the ability of microorganisms to utilize citrate as a source of carbon. A sterile inoculating loop was used to pick a portion of the isolate onto a sterile and solidified Simmons' citrate sugar slant and incubated at 37°C for 24-48 hours. This was observed for colour change. Colour change from green to blue indicates a positive result while a negative result shows no colour change (MacWilliams, 2009).

Urease test

A loopful of the bacterial isolate was inoculated into a test tube of urease broth using a sterile inoculating loop and incubated for 24 hours at 37°C. The appearance of a pink (fuchsia) (alkaline pH) phenol red colour indicated a positive urease test indicating urease production (Brink, 2010).

Estimation of viable bacterial counts

This was determined using the method described by Ephraim (2010). Aliquots of 0.5 ml of three dilution factors were introduced into 3 Petri dishes each. Molten Nutrient agar at about 45°C was added and then mixed thoroughly and allowed to set undisturbed. The set nutrient agar was later incubated at 37°C for 24 hours. These plates were examined after incubation and the number of colonies forming unit per plate was counted using the manual counting method (Karki, 2022) and the average was recorded.

Statistical analysis

Data obtained was statistically analyzed using Analysis of Variance (ANOVA) and the means were separated using the Duncan Multiple Range Test according to Sanders (1990). The statistical package used for this was SPSS 17.

RESULTS AND DISCUSSION

The number and types of microorganisms isolated from smoked Sardine, *Sardinella maderensis* samples (both preserved and unpreserved) are presented in Table 1. The microbial load of samples not preserved with lemon essential oil but packaged in polyethylene bags ranged from $10.65-25.35 \times 10^5$ CFU/g from the 1st to the 12th week of storage with *Bacillus subtilis* dominating among others like *Staphylococcus aureus*, *Lactobacillus fermentum* and *Enterobacter cloacae*. The microbial load of samples preserved with lemon essential oil decreased from 6.25×10^5 CFU/g (1st week) to 1.30×10^5 CFU/g (10th week), decreasing significantly ($p<0.05$) to the 11th and 12th week of storage where there was no visible microbial growth. While the microbial loads for preserved sardine in weeks 3 and 4 were not significantly ($p<0.05$) different from each other, they were significantly ($p<0.05$) different from weeks 6 to week 10 which were not significantly ($p<0.05$) different from one another. The weekly microbial load of unpreserved sardine increased significantly ($p<0.05$) with weeks 5 and 6 not significantly ($p<0.05$) different from each other and weeks 5 to 7 were also not significantly ($p<0.05$) different from one another. The low microbial load in sardine could be due to the fact that it was dried and smoked under a more hygienic condition and better handled post smoking.

Microorganisms isolated from stored smoked sardine (*Sardinella maderensis*) were dominated by *Bacillus subtilis* weekly all through storage. Sardine samples preserved with lemon (*Citrus limon*) essential oil had their weekly microbial loads decreasing significantly ($p<0.05$) till the 10th week while there was no growth in the 11th and 12th week. There was no significant ($p<0.05$) difference in the microbial loads in weeks 3 and 4, weeks 5 to 8 and weeks 6 to 10. This infers that smoked Sardine can be preserved with essential oil from lemon peel and stored. However, the microbial load of the unpreserved samples increased significantly ($p<0.05$) weekly throughout the storage period. Apart from weeks 1 and 2, the microbial load of preserved sardine throughout the storage period was less than the Maximum Permissible Limit (MPL) for good quality products < 10^5 CFU/g of the sample (ICMSF, 2011). The microbial loads in the unpreserved samples were extremely higher than the MPL. This confirms the use of lemon essential oil as a preservative for inhibiting microbial spoilage in smoked sardines and the fish can be kept for 10 weeks.

In all, eight bacteria were isolated from the two fish species samples with sardine having five and bonga having three microorganisms. *Bacillus subtilis* was common in both fish samples. Three of the isolates are pathogenic (*Klebsiella aerogenes*, *Staphylococcus aureus* and *Escherichia coli*), and the other three microorganisms cause spoilage (*Enterobacter cloacae, Bacillus subtilis* and *Bacillus megaterium*) while *Staphylococcus saprophyticus* and *Lactobacillus fermentum* are opportunistic pathogens and probiotic organisms, respectively. Bonga had five microorganisms isolated from it- two of which cause spoilage (*Bacillus subtilis* and *Bacillus megaterium*), two are pathogenic (*Staphylococcus aureus* and *Escherichia coli*) while *Klebsiella aerogenes* is an opportunistic pathogen.

Table 1. Microbial load and characteristics of organisms isolated from stored smoked Sardine (*S. maderensis).*

Week	S-O+N x10^5 (CFU/g)	Microorganism	S+O+Nx10^5 (CFU/g)	Microorganism
1	10.65±0.21^{g}	*Lactobacillus fermentum, Bacillus subtilis, S. aureus, Enterobacter cloacae*	6.25 ±0.35a	*Bacillus subtilis, S. aureus*
2	10.50 ±0.71^{g}	*B. subtilis, S. aureus*	5.20 ±0.28^{b}	*Bacillus subtilis*
3	15.00 ±1.41^{f}	*B. subtilis*	4.25 ±0.35^{c}	*B. subtilis*
4	19.50 ±2.21^{c}	*B. subtilis, S. saprophyticus*	4.25 ±1.06^{c}	*B. subtilis,*
5	15.75 ±1.06^{ef}	*B. subtilis*	2.25 ±0.07^{d}	*B. subtilis*
6	16.25 ±0.35^{def}	*B. subtilis*	1.80 ±0.28^{de}	*B. subtilis*
7	17.40 ±0.57^{cde}	*B. subtilis*	1.65 ±0.21^{de}	*B. subtilis*
8	18.30 ±0.42^{cd}	*B. subtilis*	1.50 ±0.14^{de}	*B. subtilis*
9	19.50 ±0.71^{c}	*B. subtilis*	1.35 ±0.21^{e}	*B. subtilis*
10	22.30 ±0.42^{b}	*B. subtilis*	1.30 ±0.41^{e}	*B. subtilis*
11	24.50 ±0.71^{a}	*B. subtilis*	0.00 ±0.00^{f}	NG
12	25.35 ±0.21^{a}	*B. subtilis*	0.00 ±0.00^{f}	NG

S-O+N= UncoatedSardine samples packaged in Polyethylene bags**; S+O+N =** CoatedSardine samples packaged in Polyethylene bags**;** NG = No Growth**.** Values denoted by different superscripts in the column differ significantly [$p < 0.05$].

Table 2. Microbial load and characteristics of organisms isolated from stored smoked Bonga (*E. fimbriata)*

Week	B-O+N x10^5 (CFU/g)	Microorganism	B+O+Nx10^5 (CFU/g)	Microorganism
1	12.50± 0.71^{a}	*B. subtilis, Klebsiella aerogenes, E. coli*	9.25 ± 0.35^{a}	B. subtilis
2	14.50 ± 0.71^{f}	*B. subtilis, E. coli*	6.75 ± 0.35^{b}	*B. subtilis*
3	14. 50± 0.71^{f}	*B. subilis, B. megaterium*	6.75± 0.35^{b}	*B. subtilis*
4	15.50 ± 0.71^{ef}	*B. subtilis, S. aureus*	4.75 ± 035^{c}	*B. subtilis*
5	16.45 ± 0.07^{e}	*B. subtilis*	3.75± 0.35^{d}	*B. subtilis*
6	16.20 ± 0.28^{ef}	*B. subtilis*	2.75± 0.35^{ef}	*B. subtilis*
7	17.00± 0.71^{de}	*B. subtilis*	3.05 ± 0.35^{e}	*B. subtilis*
8	18.65 ± 0.21^{cd}	*B. subtilis*	2.75 ± 0.35^{ef}	*B. subtilis*
9	19.25 ± 0.35^{c}	*B. subtilis*	2.40 ± 0.28^{ef}	*B. subtilis*
10	21.50± 2.12^{b}	*B. subtilis*	2.20 ± 0.28^{f}	*B. subtilis*
11	26.25± 0.35^{a}	*B. subtilis*	0.00 ± 0.00^{g}	NG
12	26.25 ± 0.35^{a}	*B. subtilis*	0.00 ± 0.00^{g}	NG

B-O+N= UncoatedBonga samples packaged in Polyethylene bags; **B+O+N =** CoatedBonga samples packaged in Polyethylene bags; NG = No Growth. Values denoted by different superscripts in the column differ significantly [$p < 0.05$].

The number and types of microorganisms isolated from smoked Bonga, *Ethmalosa fimbriata* samples (both preserved and unpreserved) from week 1 to week 12 are presented in Table 2. The microbial load of samples not preserved with lemon essential oil but packed in polyethylene bags ranged from 12.50 − 26.25x10^5 CFU/g from the 1st to the 12th week of storage with *Bacillus subtilis* dominating among others like *S. aureus*, *B. megaterium*, *E. coli* and *Klebsiella aerogenes*. The weekly microbial load of samples preserved with lemon essential oil ranged from 9.25−2.20x10^5 CFU/g, decreasing significantly ($p<0.05$) to the 11th and 12th week of storage when there was no visible microbial growth. There was no significant ($p<0.05$) difference in the microbial load in weeks 6 to 9, as well as in weeks 6, 8 and 10.

A total of five bacterial isolates were isolated from the unpreserved samples, even though *Bacillus subtilis* was the only isolate present in the preserved samples in week 2, it was isolated in the company of *Staphylococcus aureus* and *S. saprophyticus* in the unpreserved samples till week 4. The lemon essential oil also showed high antimicrobial activity on bonga (*Ethmalosa fimbriata*) as it was able to reduce the microbial load of the preserved samples from 9.25x10^5 CFU/g in week1 to 2.20x10^5 CFU/g in week10. There was no significant difference ($p<0.05$) in the microbial load in weeks 2 and 3. Also, there was no significant ($p<0.05$) difference in the microbial load of the preserved samples of both bonga and sardine from the 6th to the 10th. This infers that preserving bonga and sardine with lemon essential oil reduces the microbial load below

Table 3. Morphological and Biochemical Characterization of Bacterial Isolates obtained from stored smoked Bonga (*E. fimbriata*) and Sardine (*S. maderensis*) during storage.

ID	Gram reaction	Spore staining	Capsule staining	Catalase	Coagulase	Motility	Indole	Oxidase	Citrate	Urea
F_1	GNB	-	+	+	-	-	-	-	+	+
F_2	GNB	-	-	+	-	+	-	-	+	-
F_3	GPC	-	-	-	+	-	-	-	-	-
F_4	GPC	-	-	-	-	-	-	-	-	-
F_5	GNB	-	-	+	-	+	+	-	-	-
F_6	GPB	+	+	+	-	+	+	-	-	-
F_7	GPB	-	-	-	-	-	-	+	-	-
F_8	GPB	-	+	-	-	-	-	+	-	-

-:Negative, + :Positive GNB-Gram Negative Bacilli A- Acid Production GPB- Gram Positive Bacilli GPC- Gram Positive Cocci.

Table 3. Contd.

ID	H_2S	Methyl-red	Voges proskeur	Glucose	Lactose	Sucrose	Mannitol	Probable microorganism
F_1	-	-	+	A	A	A	A	*Klebsiella aerogenes*
F_2	-	+	-	A	-	-	A	*Enterobacter cloacae*
F_3	-		+	A	A	A	A	*Staphylococcus aureus*
F_4	-	+	-	A	-	-	-	*Staphylococcus saprophyticus*
F_5	-	+	-	A	A	-	-	*Escherichia coli*
F_6	-	+	-	A	-	-	-	*Bacillus subtilis*
F_7	-	+	-	A	A	-	-	*Lactobacillus fermentum*
F_8	-	+	-	A	A	-	-	*Bacillus megaterium*

-: Negative, +:Positive GNB-Gram Negative Bacilli A- Acid Production GPB- Gram Positive Bacilli GPC- Gram Positive Cocci.

the recommended MPL. Lemon essential oil can be applied to smoked bonga and sardine to reduce the microbial load, as it did to these samples after storage for 6 weeks and the samples had microbial loads that were within MPL for food products. This corroborates the findings of Ayeloja (2016) who achieved the MPL in smoked catfish preserved with spices on the 56th day (8th week). It can thus be stored for 11 or 12 weeks to completely eradicate the spoilage organisms as seen in this study.

The oil completely eradicated the microorganisms in the 10th week of storage as there was no microbial growth observed. This was evident in the unpreserved samples as the load increased weekly from $12.50x10^5$ (week 1) to $26.25x10^5$ (week 12). The microbial flora of bonga was only *Bacillus subtilis* throughout the storage period (for the preserved samples) while the unpreserved sample had *Escherichia coli, Klebsiella aerogenes, Bacillus megaterium, Staphylococcus aureus* alongside *Bacillus subtilis* till the 4th week before it remained only *Bacillus subtilis*. This difference showed the antimicrobial effectiveness of the lemon essential oil as it was able to gradually reduce the fish microbial load weekly. It was able to perform this antimicrobial action with the help of the chemical compounds (D-Limonene, 2. Decenal, Squalene) that were present in it. This corroborates Dike-Ndudim *et al*. (2014) who reported that microflora of fish (fresh or smoked) is usually from the genera of *Bacillus, Staphylococcus, Klebsiella, Penicillium* etc. The microbial loads were still lower than those of Ikpebivie and Amakoromo (2013) and higher than of Olayemi *et al*. (2011). The microbial load in the preserved samples of both fish was higher than that reported by Adetimehin *et al.* (2019) for the first four weeks of storage, though bonga in the 5th week was still higher than this finding, it was still within the MPL of microorganisms. This variation may be particular to the fish species, and the environment it was caught, sold or smoked.

The colonial characteristics of microorganisms isolated from the fish samples on nutrient agar as shown in Table 3 revealed that seven out of the eight bacteria isolates have a round shape and wet consistency. The colonial size ranged from 0.5 to 4 mm and the colours observed include creamy white, grey and white. The morphological and biochemical characteristics of the isolates presented in Table 3 revealed that three of the isolates were Gram-negative *Bacilli*, two Gram-positive cocci and three Gram-positive *Bacilli*. Four isolates were catalase positive; three were motile and all microorganisms produced acid during glucose fermentation. The eight isolates are *Bacillus*

subtilis, Klebsiella aerogenes, Escherichia coli, Bacillus megaterium, Staphylococcus aureus, Enterobacter cloacae, Lactobacillus fermentum and *Staphylococcus saprophyticus*.

Conclusion

This study discovered that lemon essential oil reduced spoilage microorganisms significantly in clupeids [(*Ethmalosa fimbriata* –Bonga) and (*Sardinella maderensis* –Sardine)]. This was evident in its ability to drastically reduce the number of microorganisms found on the stored preserved fish samples compared to the unpreserved ones. It has also been confirmed that lemon essential oil is an effective natural preservative for smoked clupeids. Lemon peel essential oil reduced the number of microorganisms on smoked clupeids after 6 weeks of storage till there was no microbial growth observed.

Recommendations

It is therefore recommended that other citrus essential oils sweet orange (*Citrus sinensis*), tangerine (*Citrus reticulata*), and lime (*Citrus aurantifolia*) be experimented with to see if similar effects will be produced. Packaging preserved fish in polyethylene bags before being placed in a box is also recommended as a way of keeping out microorganisms that can spoil fish and make the nutrients in it inaccessible to consumers.

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