EFFECTS OF SALTS ON PRESERVATION AND METABOLIC ACTIVITIES OF FISH AND MEAT MICROFLORA

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Abstract

Foods usually carry a mixed population of microorganisms derived from both the natural microflora of the food plant or animal and those introduced during handling, processing, and storage. Salt is a widely used additive and preservative, which, influences microorganisms in different concentrations. This study aims to determine the effect of salts on food preservation and metabolic activities of food microflora. Two food samples (raw fish and raw lean meat) were investigated. Sodium chloride (NaCl), Potassium chloride (KCl) and Calcium chloride ($CaCl_2$) were grouped into varying concentrations of 2, 2.5 and 4.5% respectively. The food samples were pulverized and salted according to these concentrations and an unsalted group served as the control. Storage was for fifteen days with samples analysis on days 0, 7 and 15. The first category of food sample was preserved under room temperature (28 ± 2 °C), with the second and third under refrigeration (4 ± 2 °C) and freezer (-18 ± 2 °C) temperature respectively. Total protein and Lipid Peroxidation were assayed during the duration of storage. Organisms isolated from the samples were identified based on biochemical and cultural characteristics to include Staphylococcus aureus, Escherichia coli, and species of Pseudomonas, Klebsiella, Enterobacter, Aspergillus, Moulds, and Yeasts, with Bacillus spp mostly predominant. There was a decrease in microbial population along concentration and temperature gradient, the population, however, increased along the duration of storage. The total protein decreased while lipid peroxidation increased along the duration of storage. NaCl showed the highest efficiency in preservation, with KCl showing the least efficacy. Freezer temperature (-18 ± 2 °C) and 4.5% salt concentrations were the most effective. Salts alone at concentrations acceptable in food cannot effectively control an initial high load of natural and contaminant microflora in raw foods. Use of salts in synergy with other preservatives/ methods like refrigeration will drastically reduce the activities of food microflora whilst enhancing the shelf-life of these foods.

Keywords: *Salts, food preservation, metabolic activities, lipid peroxidation, Microflora*

Introduction

Food is essential for the growth and survival of man but becomes harmful through the presence and action of spoilage organisms and foodborne pathogens (ICMSF, 1996). Microorganisms are known to be ubiquitous in nature; they can be found almost everywhere including foods (Hall, 1997). Foods are rarely sterile because these organisms are present in the air surrounding the food, water, and soil from where the food plants and animals are harvested (ICMSF, 1996). Foods usually carry a mixed population of microorganisms derived from both the natural microflora of the original plant or

animal and those introduced during harvest/slaughter and subsequent handling, processing, and storage (Hall, 1997). Quality deterioration caused by these microorganisms may include a wide range of types of spoilage that are not desirable commercially, because they limit shelf life or lead to quality complaints, but are safe from a public health point of view (ICMSF, 1996). The presence or growth of infectious or toxigenic microorganisms (foodborne pathogens) represents the worst forms of quality deterioration because they threaten the health of the consumer (ICMSF, 1996). ICMSF, (1996) observed that despite the extensive scientific progress and technological developments achieved in recent years, food safety problems continue to exist and may actually increase in the future. Both intrinsic and extrinsic factors act synergistically to enhance or retard the growth of microorganisms in food (Jay *et al.,* 2005).Salt is a widely used additive and preservative, enhancing the flavour (Silva *et al.*, 2003) and improving water adsorption in foods (Lawrence *et al.*, 2003). A high salt concentration generates changes in cellular metabolism because of its osmotic effect, which, influences microorganisms in different concentrations. Bautista *et al* (2007) and Blesta *et al* (2008) reported that the salts, NaCl, KCl and $CaCl_2$ or their combination drastically reduced water activity in meat. Addition of salts leads to a sudden onset of plasmolysis, which causes inhibition of nutrient uptake, DNA replication and triggers an increase in the ATP levels of cells, which further leads to the inhibition of macromolecular biosynthesis (Csonka, 1989). According to Albarracin *et al.,* (2010), salt is one of the most preferably used additives in food industries because of its low cost and varied properties. Its unique properties lie in the preservative and antimicrobial effect as a direct consequence of the capacity of sodium chloride to reduce water activity values (Albarracin *et al.*, 2010). Salt, which interacts with other physicochemical parameters, have been used as a preservative to inhibit the growth of microorganisms while maintaining the shelf-life of food products (Andres *et al.*, 2005). Furthermore, sodium chloride influences the activity of different proteases and proteins such as Ca-dependent protease; Cathepsin D and Cathepsin L (Armenteros *et al.*, 2009). An increase in the concentration of NaCl has been reported to decrease protease activities and thus prevent the spoilage of meat (Armenteros *et al.*, 2009).

Food-borne illnesses that occur from consuming contaminated food with pathogenic bacteria have been of serious public health concern all over the world. Food-borne illnesses associated with *E. coli 0157:H7, Staphylococcus aureus, Salmonella enteritidis, Salmonella typhi* and *Listeria monocytogenes,* is a major concern (Beuchat 1996, Hall, 1997 and Farber, 2000). The result also informs of the need to wash raw food products in potable water to reduce microflora before preservation because, in the presence of a high initial microbial load, preservation may virtually be impossible. This work aims to determine effects of salts on metabolic activities of food (meat and fish) microflora as purchased from the market

Materials and Methods

Sampling points

Fish and Meat samples were collected from three different locations in a local market in Ota (Oja-Ota). Ota is located in Ogun state, Nigeria (7.9452°N, 4.7888°N). This market was chosen because it is the major one in town and many vendors patronize the market for sales. Food grades of NaCl, KCl, and $CaCl_2$ were obtained from reputable vendors.

Collection of Samples

Major vendors of fresh fish and meat were identified in the market. Samples were randomly obtained from six vendors, three (3) each for raw fish and meat samples. All the samples were collected in polyethylene bags as sold and transported in cold pack (2 ± 2°C) to the Microbiology Laboratory of Covenant University, Ota for analysis within 30 minutes-1hour after collection.

Sample Preparation

A sterile knife was used to remove fatty portions of the meat to obtain lean meat. Twenty-five grams (25g) portions of the samples were minced in a laboratory blender (Model 400 EVO, Stomacher) and homogenized in 225ml of sterile peptone water. The resultant homogenate was diluted 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. From the appropriate dilution, 0.2 ml were plated in duplicate onto the different media using the spread plate technique. Nutrient agar, Eosin Methylene blue agar, and Potato Dextrose agar were inoculated for isolation of bacteria and fungi and for Total aerobic plate count, Coliform count and fungal count respectively. Mannitol salt agar was used for isolation and counting of *Staphylococcus aureus.*

Isolation and Enumeration of Bacteria and Fungi

All inoculated plates were incubated at 37°C for 24- 48 h to obtain viable bacterial counts, except, however, Saboraud Dextrose agar plates that were incubated at laboratory room temperature of 28 ± 2 °C for 72 h. Colonies were counted at the expiration of incubation period using the colony counter (Gallenkamp, England). Counts were expressed as colony forming units per ml of sample homogenate (cfu/ml). Characteristic discrete colonies on the different media were isolated and purified by repeated sub-culturing on nutrient agar. Pure cultures were stored on agar slants at 4°C for further characterization.

Coliform Test

The standard method of Speck, (1976) as described by Oranusi *et al.,* (2013) was adopted. One gram of each sample was transferred to a sterile test tube containing Lactose broth and inverted Durham tubes. Incubation was for 24-48 h at 37°C before tubes were checked for gas production. This was the presumptive test. A loop full of inoculum from gas positive tubes was streaked onto Eosin methylene blue (EMB) agar plates and incubated at 37°C and 45°C for 24 hrs. Following incubation, colonies which formed bluish black colonies with green metallic sheen, and reddish/ brown colonies were noted and isolated on agar slants (confirmatory test). Also, colonies showing metallic sheen on EMB were subcultured into tubes of lactose broth (single strength) and incubated at 37°C. The tubes were observed after 24 h for gas production (completed test). The growth of coliform at 45°C incubation was noted as faecal coliform.

Identification of Isolates

The bacterial isolates were identified based on standard methods of Speck (1976); Cowan, (1985). Isolates were Gram stained and specific biochemical tests performed to include Catalase activity, Sugar utilization, Oxidase test, Indole test, Urease test, Methyl red and Voges Proskauer tests, Coagulase activity, Citrate utilization and Motility test. API bioMérieux© kit was employed for additional identification of the bacterial isolates. Fungal isolates were identified based on their macroscopic and microscopic characteristics employing cultural presentation, pigment production on media, hyphal and spore characteristics and with reference to standard identification keys and atlas for fungal identification (Fawole, 1986; Tsuneo, 2010).

Preservation Study

The samples were minced with a laboratory blender (Model 400 EVO, Stomacher). Three different weights of 2g, 2.5g, and 4.5g of the salts NaCl; KCl and $CaCl_2$ were well mixed with 100g of samples for preservation. The salted samples were transferred into sterile tubes with lids and appropriately labeled. A group of unsalted samples served as the control. Storage of the samples was under three varying conditions namely:

- Room Temperature at 28±2°C
- Refrigeration Temperature at 4±2°C
- Freezing Temperature at -18±2°C

Samples were stored for fifteen days during which assay for microbial profile and metabolic activities was conducted on day 0, day 7 and day 15.

Assay for Metabolic Activities and Microbial Profile during Storage

Metabolic activity was determined by total protein and lipid peroxidation. The microbial profile was evaluated following the procedure earlier described in enumeration and identification of isolates. Determination of protein was by biuret method following the description of Sapan and Lundblad (2015). Egg albumin served as standard and the absorbance was read at 450nm using a spectrophotometer (Model G105 UV-VIS Genesys, ThermoFisher Scientific). Lipid peroxidation was evaluated by TBARS assay measuring thiobarbituric acid reactive substances (Malondialdehyde-MDA). Aliquot of 1 ml of sample homogenate was combined with 2 ml of Thiobarbituric acid (TBA) reagent and mixed thoroughly. The solution was incubated in boiling water bath for 15 minutes. After boiling, the tubes were immediately placed under a running tap to cool. The tubes were then centrifuged at 1000 rpm for 10 minutes and the absorbance of the clear supernatant was read against a blank (all reagent minus sample homogenate) at 535nm. TBARS content was calculated using 1.56×10^5 $M^{-1}cm^{-1}$ which is the extinction coefficient of MDA-TBA complex at 535 nm.

Results

Table 1 shows the results of samples before preservation. It reveals initial high microbial load in all the samples. Table 2 presents the microbial loads during storage. It shows an increase along the duration of storage and along the salt concentration with more increase at room temperature storage when compared to refrigeration and freezing temperature. Table 3 shows the TBARS values (molMDA/g) of samples during

storage. The table reveals an increase in lipid peroxidation along the storage duration, temperature and salt concentrations. The protein contents were shown to decrease along salt concentration levels and duration of storage

Table 1: Mean microbial load (cfu/ml), lipid peroxidation and protein of samples before preservation

Analysis	Meat Samples			Fish Samples (cfu/ml)		
	1	2	3	1	2	3
TAPC	7.2×10^6	7.8×10^6	1.1×10^8	2.0×10^8	1.7×10^8	5.9×10^6
TCC	5.0×10^4	1.4×10^5	1.5×10^4	1.8×10^5	4.1×10^5	1.2×10^5
TFC	1.6×10^5	6.2×10^4	7.0×10^4	5.9×10^6	8.0×10^4	1.4×10^5
Lipid peroxidation (molMDA/g)	0.011			0.099		
Protein content (mg/g)	0.588			1.330		

Key: TAPC: Total Aerobic Plate Count; TCC: Total Coliform Count; TFC: Total Fungal Count

Table 2: Mean total microbial count (cfu/ml) during preservation at 28±2°C room temperature

Concentration / TAPC	Meat Samples								Fish Samples							
	NaCl		KCl		$CaCl_2$		Control		NaCl		KCl		$CaCl_2$		Control	
	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15	Day 7	Day 15
2.0	2.2×10^9	4.8×10^9	1.3×10^9	4.3×10^9	1.1×10^9	1.1×10^9	1.4×10^{10}	3.4×10^{10}	3.4×10^9	6.0×10^9	5.2×10^9	7.2×10^9	5.0×10^9	5.9×10^9	2.0×10^{10}	4.2×10^{10}
2.5	1.8×10^9	4.0×10^9	9.8×10^8	4.1×10^9	8.2×10^8	8.2×10^8	-	-	2.8×10^9	5.8×10^9	5.0×10^9	6.3×10^9	4.8×10^9	5.5×10^9	-	-
4.5	6.0×10^8	3.9×10^8	8.9×10^8	4.0×10^9	6.3×10^8	7.9×10^8	-	-	2.5×10^9	4.5×10^9	3.7×10^9	5.8×10^9	4.3×10^9	4.9×10^9	-	-
Total Coliform Count																
2.0	1.9×10^7	3.2×10^8	1.1×10^7	4.5×10^8	9.9×10^7	2.5×10^8	2.1×10^8	2.1×10^9	4.5×10^8	4.5×10^8	5.1×10^8	7.9×10^8	3.1×10^8	3.7×10^8	2.0×10^9	3.5×10^9
2.5	1.6×10^7	2.8×10^8	6.6×10^6	4.0×10^8	9.2×10^7	2.1×10^8	-	-	4.1×10^8	4.3×10^8	4.9×10^8	7.5×10^8	2.7×10^8	3.1×10^8	-	-
4.5	5.0×10^6	1.9×10^7	5.2×10^6	3.4×10^7	7.7×10^7	1.9×10^7	-	-	3.2×10^8	3.8×10^7	4.4×10^8	6.8×10^8	2.4×10^8	2.7×10^8	-	-
Total Fungal Count																
2.0	5.9×10^6	6.5×10^7	6.2×10^6	7.3×10^7	4.8×10^6	5.1×10^7	1.1×10^8	3.2×10^8	2.0×10^7	2.0×10^7	3.2×10^7	3.2×10^7	8.2×10^6	8.2×10^6	6.5×10^8	6.5×10^8
2.5	5.2×10^6	6.2×10^7	5.8×10^6	6.8×10^7	4.5×10^6	4.7×10^7	-	-	9.1×10^6	9.1×10^6	2.8×10^7	2.8×10^7	7.3×10^6	7.3×10^6	-	-
4.5	4.9×10^5	5.9×10^7	5.3×10^6	6.2×10^7	4.1×10^6	4.2×10^7	-	-	8.7×10^6	8.7×10^6	1.6×10^7	1.6×10^7	6.8×10^6	6.8×10^6	-	-

Table 3: Mean TBARS values (molMDA/g) of samples at days 7 and 15

Concentration / Day 7	Meat Samples												Fish Samples											
	NaCl			KCl			$CaCl_2$			Control			NaCl			KCl			$CaCl_2$			Control		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
2.0	0.031	0.029	0.026	0.045	0.035	0.031	0.027	0.031	0.028	0.042	0.036	0.045	0.223	0.211	0.202	0.250	0.230	0.223	0.223	0.222	0.215	0.305	0.276	0.269
2.5	0.027	0.024	0.026	0.039	0.032	0.027	0.027	0.031	0.025	-	-	-	0.195	0.203	0.198	0.241	0.224	0.219	0.195	0.217	0.207	-	-	-
4.5	0.024	0.019	0.018	0.028	0.026	0.022	0.019	0.025	0.020	-	-	-	0.187	0.198	0.193	0.212	0.217	0.208	0.187	0.211	0.199	-	-	-
	TBARS value of samples at day 15																							
2.0	0.061	0.048	0.031	0.075	0.062	0.052	0.067	0.053	0.044	0.084	0.079	0.068	0.330	0.315	0.308	0.517	0.387	0.354	0.298	0.276	0.241	0.424	0.410	0.397
2.5	0.054	0.042	0.039	0.062	0.051	0.049	0.059	0.047	0.041	-	-	-	0.323	0.306	0.299	0.394	0.326	0.348	0.274	0.253	0.230	-	-	-
4.5	0.036	0.037	0.033	0.048	0.046	0.046	0.032	0.041	0.039	-	-	-	0.311	0.297	0.282	0.363	0.312	0.332	0.265	0.240	0.222	-	-	-

Key: A= 28±2°C; B= 4±2°C; C= -18±2°C

Table 4: Mean total protein value (mg/g) of samples at day 7 and 15

Concentration / Day 7	Meat Samples												Fish Samples											
	NaCl			KCl			$CaCl_2$			Control			NaCl			KCl			$CaCl_2$			Control		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
2.0	0.164	0.222	0.227	0.097	0.154	0.154	0.025	0.138	0.138	0.065	0.089	0.089	0.440	0.452	0.452	0.304	0.317	0.317	0.187	0.205	0.205	0.102	0.111	0.111
2.5	0.183	0.269	0.269	0.106	0.178	0.178	0.073	0.135	0.135	-	-	-	0.588	0.594	0.594	0.359	0.393	0.393	0.224	0.256	0.256	-	-	-
4.5	0.193	0.323	0.323	0.256	0.291	0.291	0.083	0.319	0.319	-	-	-	0.743	0.755	0.755	0.762	0.781	0.781	0.725	0.751	0.751	-	-	-
Total protein value of samples at day 15																								
2.0	0.109	0.105	0.103	0.099	0.095	0.095	0.121	0.124	0.124	0.039	0.039	0.041	0.285	0.283	0.290	0.276	0.278	0.278	0.156	0.162	0.162	0.058	0.065	0.065
2.5	0.134	0.127	0.127	0.112	0.112	0.114	0.152	0.151	0.151	-	-	-	0.524	0.521	0.522	0.330	0.339	0.351	0.175	0.181	0.185	-	-	-
4.5	0.150	0.145	0.149	0.136	0.136	0.136	0.223	0.223	0.223	-	-	-	0.636	0.636	0.636	0.342	0.342	0.342	0.268	0.268	0.268	-	-	-

Key: A= 28±2°C; B= 4±2°C; C= -18±2°C

Discussion

This study investigated the effects of salts on food preservation and the metabolic activities of food microflora. Salting, like all other preservation methods, aim to maintain the nutritional characteristics of the food and ensure a longer shelf life. The different preservation methods applied to foods and the shelf life ultimately determines their commercial viability. Results showed that the diverse microbial species isolated from both fish and meat prior to addition of preservatives include *Pseudomonas spp, Staphylococcus aureus, Proteus spp, Escherichia coli, Klebsiella spp, Enterobacter spp, Aspergillus spp* and *Mucor spp.* Microbiological quality of food samples is often assessed by the total microbial load determined using the total aerobic plate count, total coliform count and total fungal count. The microbial counts gradually increased with storage, this could be attributed to high moisture and nutrient contents of the samples which could promote the growth of the organisms (Gandotra, 2017).

The presence of these diverse species of organisms on the food samples could be attributed to contamination from the environment. Meat and fish are rich in nutrients required for growth and proliferation of microorganisms. *Proteus* and *Pseudomonas spp* are common environmental contaminants of food and food products. Species of *Klebsiella, Enterobacter, and Escherichia coli* are members of Enterobacteriaceae associated with the gastrointestinal tract, the occurrence of these organisms in the fish and meat samples may be attributed to poor sanitary practices in slaughtering, processing, and packaging prior to purchase (Famurewa *et al.,* 2017). *E. coli* are normal flora of the human and animal intestine, it is an indicator organism of faecal contamination, and pathogenic strains have been identified as a leading cause of foodborne illness all over the world. *E. coli* has previously been isolated from meat samples (Hussein, 2007). Its presence in the samples could be associated with poor sanitary practices in the handling of the meat and fish samples, this call for public health concerns. *Staphylococci* are normal flora of man and animals, its presence in these samples collaborate with the food handlers and normal flora of the animals. The presence of *Staphylococcus aureus* also raises some concerns as toxigenic strains have been associated with foodborne infection (Hennekinne *et al.*, 2012; Ji-Yeon *et al.*, 2013).

Results indicated that the TBARS value of the fish samples was higher than those of the meat samples before treatment. The TBARS value of the samples increased as storage time increased. This was expected and corroborates findings by Farbod (2015) who reported that TBARS values increase with storage time. In the meat samples, those treated with NaCl and stored at room temperature potentially retarded the generation of the reactive species the most. The KCl treated meat samples exhibited the highest increase in TBARS values between day 7 and 15. Although it has been earlier suggested that TBARS value increases with storage time, there was an unexpected increase among meat

samples treated with 2% NaCl between day 7 and 15. In the fish samples, the $CaCl_2$ treated samples had minimal variation in TBARS increment compared to the NaCl and KCL treated samples. Generally, the most retardation of MDA generation was observed in samples stored at freezing temperatures. This corroborates findings by Aydin (2014) who reported that low freezing temperatures result in low oxidation levels. Freezing is also a good preservation method known to prevent microbial proliferation. The TBARS of the samples were lower than those of the control during the storage period which is indicative of the preservative potential of the salts. However, the increase in the TBARS values during storage may also have been mediated by the salts themselves as reports have shown that salts such as NaCl possess prooxidant activity and have been implicated in lipid oxidation in meat and seafood (Mariutti and Bragagnolo, 2017).

Results showed that the total protein of the samples decreased with storage after salt treatment, with the fish samples showing a higher rate of protein decrease compared to the meat samples. It has been reported that this pattern of total protein decrease may be a consequence of denaturation (Gupta, 2017) there was a sharp decrease in total protein among sample stored under refrigeration and freezing conditions. This corroborates findings by Gandotra *et al.,* (2014) that low storage temperatures enhance protein denaturation. However, it was observed that the KCl and $CaCl_2$ treated meat samples stored at room temperature showed an increase in total protein value during storage. In both the meat and fish samples, the total protein values were generally higher than those of their respective control. This may be attributed to strong salting out effects on the proteins (Thorarinsdottir *et al.,* 2004).
Sodium Chloride (NaCl) exhibited the highest efficacy in the preservation of food samples overall, while Potassium Chloride (KCl) showed the least efficacy.

Conclusion

Food quality remains an important parameter for good health thus must be in check. To ensure freshness of foods, preservative methods like salting are often employed, this present study showed the presence of certain organisms associated with fresh fish and meat. There was an increase in the microbial population in the selected foods along the duration of preservation, with more increase at room temperature; the microbial counts, however, fell within permissible limits for raw food. The 4.5% salt concentration and freezing was the most effective preservation method. The decrease in total protein along the duration of preservation shows how essential nutrients are depleted due to the metabolic activities of organisms. However, an increase in total protein along salt concentration and temperature gradient shows how the reduction and inhibition of certain organisms slightly maintain the nutrients in foods.The increase in lipid peroxidation along storage duration could be indicative of microbial, enzymatic and prooxidant activities in the salts. Storage temperature and salt concentration ultimately determine the rate at which lipid peroxidation occurred. In this study, NaCl showed the highest efficacy in preservation, while freezer

temperature and 4.5% salt concentration were the most effective, Preserving food with a high initial level of microbial load, makes preservatives ineffective and preservation difficult. It is advanced that for effective preservation of raw fish and meat products, initial levels of microbial contaminants must be reduced to the barest minimum.

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