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Effects of Chemical Hurdles and Packaging Materials on Fungal Load and Distribution in *Kilishi* during Ambient Storage

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Abstract:
Commercial sample of Kilishi was collected from Agadasawa in Kano State Nigeria. Laboratory prepared samples were produced by skilful slicing and drying (sun) of beef, followed by dipping into condiment made from defatted groundnut, spices, seasoning and chemical hurdles (sucrose, citric acid and sodium benzoate). The dipped slices were finally sun-dried and roasted on a glowing charcoal. Samples were divided into three and each of the portion was packaged into High Density Polyethylene (HDPE), aluminium foil and brown paper. Fungal loads and distribution in the laboratory prepared and commercial samples were studied for twelve weeks under ambient storage (30 ± 8^0C). Increase in fungal count was observed in all the samples during storage. Highest counts were recorded in market sample and least counts were recorded in Kilishi sample treated with 4% sucrose, 0.1% citric acid and 0.1% sodium benzoate. Kilishi samples packaged in HDPE were found to have better fungal quality. Mucor, Rhizopus stolonifer, Aspergillus fumigatus and Aspergillus flavus were isolated from all the samples. Mucor, Rhizopus stolonifer succeeded the growth of Aspergillus fumigatus and Aspergillus Flavus in all the laboratory prepared samples before the end of the twelve weeks ambient storage. Incorporation of 4% sucrose, 0.1% citric acid and 0.1% sodium benzoate and use of HDPE as packaging material in tradition production of Kilishi will provide product with improved microbial quality.

Keywords: *Kilishi, hurdles, fungal load, ambient storage, packaging*

1. Introduction

Kilishi is a sun dried traditional meat product principally made from beef (Isah and Okubanjo, 2012). It is seasoned with salt, spices and defatted groundnut, then briefly roasted (Fonkem *et al.,* 2010).Hurdle technology is a process of rendering food to be free from spoilage and pathogenic microorganisms by the combination of one or more preservation methods. The spoilage and pathogenic microorganisms have to pass through these individual approaches called "hurdles" for maintaining their activity in food products (Subha, 2013).

The microbial stability and safety of most foods is based on a combination of several factors (hurdles), which should not be overcome by the microorganisms present (Leistner, 1994). Hurdle application of different treatments offers synergistic advantage compared to separate using of the individual treatment (Bazhal*et. al.,* 2003). Combining inhibitory factors can result in a significant improvement in securing microbial safety and stability as well as the sensory and nutritional quality of foods (Juneja, 2003). These include manipulation of factors such as temperature, water activity and acidity, as well as processes such as gas packaging and high pressure processing. The aim is to interfere with several different mechanisms within microorganisms simultaneously. This multi-targeted approach allows effective use of mild techniques (IFIS, 2005).

In hurdle technology, combination treatments are applied because it is expected that the use of combined preservative factors will have greater effectiveness at inactivating microorganisms than the use of any single factor. However, recent studies show that the combination of preservation factors can have unexpected antimicrobial activity (Leistner, 2011). The combined use of several preservation methods, possibly physical and chemical, or a combination of different preservatives is an age-old practice. It has been commonly applied by the food industry to ensure food safety and stability (Lee, 2004). The mechanisms by which the combination of factors, or hurdle concept, works is that; when two target microorganisms ***a*** and ***b*** can grow when preservation methods ***X*****,** ***Y*****,** or ***Z*** are used as individual hurdles. Then, if *X* and *Y* are combined, the growth of ***a*** is arrested, and when ***X*****,** ***Y*****,** and ***Z*** are used in combination, both microorganisms fail to grow (Bibek, 2005)**.**

Some preservatives at high concentrations represent chemical hazards; a combination of chemical preservatives with other preservation methods is useful. Proper application of combined methods gives stable products, prevents the undesired side-effects of each individual treatment, saves energy and lowers the required concentration of added preservatives (Pokorn, 1994). Hurdle technology proved very successful, since an intelligent combination of hurdles secures the microbial stability and safety as well as the sensory, nutritive, and economic properties of a food (Leistner, 1994).

Various biochemical changes and micro-organisms are associated with meat, during the process of slaughter, processing and preservation (Odey*et. al.,* 2013). Okonko*et. al.,* (2013) reported that *Kilishi* suffer serious contamination during processing and handling, the rate of spoilage is also very high during raining season (Fonkem*et. al.,* 2010). *Kilishi* has low microbiological quality and poor hygienic practices. The possible sources of contamination during *Kilishi* processing include contaminated raw meat, contaminated water, using contaminated utensils, addition of contaminated ingredients and poor personnel hygiene. Sun drying can also expose the sliced meat to insects and airborne microorganisms (Okonko*et. al.,* 2013).The species of *Staphylococcus, coliforms* and *Aspergillus spp* were recorded to have maximum percentage occurrence in *Kilishi* (Egbebi and Seidu, 2011).Daminabo*et. al.,* (2013) recorded high enterococcal count in *Kilishi* and he related that to lack of awareness in food safety and poor handling practices pre and post processing. Good precautionary measures must be taken to prevent or greatly reduce *Kilishi* contamination by micro-organisms during transportation of carcass and preparation of the product (Okonko*et. al.,* 2013). The extent to which *Kilishi* is contaminated by microorganisms depends on the level of hygiene and sanitation of persons involved and material used in the production chain. The degree of humidity of a food material is responsible for the initiation or inhibition of the growth of micro-organisms (Fonkem*et. al.,* 2010).

2. Methodology

2.1. Sample Collection

Freshly prepared *Kilishi* was collected from *Agadasawa* in Kano metropolis, Kano State-Nigeria. To avoid contamination, the collected sample was wrapped in HDPE and aseptically transported to laboratory. The collected sample was divided in three, each of the portions was packaged into HDPE, aluminium foil and brown paper, and stored under ambient temperature (30 ± 8^0C) for period of twelve weeks. Samples were withdrawn from market and laboratory prepared samples and subjected to microbiological analyses at two weeks' interval.

2.2. Procurement of Raw Material for Kilishi Production

Beef was purchased from Kano central abattoir. Ginger, Cloves, Black Pepper, Hot Pepper, Sweet Pepper, onion, curry, salt, seasoning and peanut cake were purchased from *Kurmi* Market in Kano.

2.3. Production of Kilishi

The table below 2 provides recipe for the production of *Kilishi* condiment.

Ingredients	**Quantity (g)**
Ginger	17.9
Cloves	1.3
Black Pepper	2.5
Hot Pepper	5.3
Sweet Pepper	11.0
Onion	12.5
Curry	3.7
Salt	23
Seasoning (Maggi)	53.5
Peanut cake	469.3

Table 1: Recipe for Production of Kilishi Condiment
Source: Badauet al. (1997)

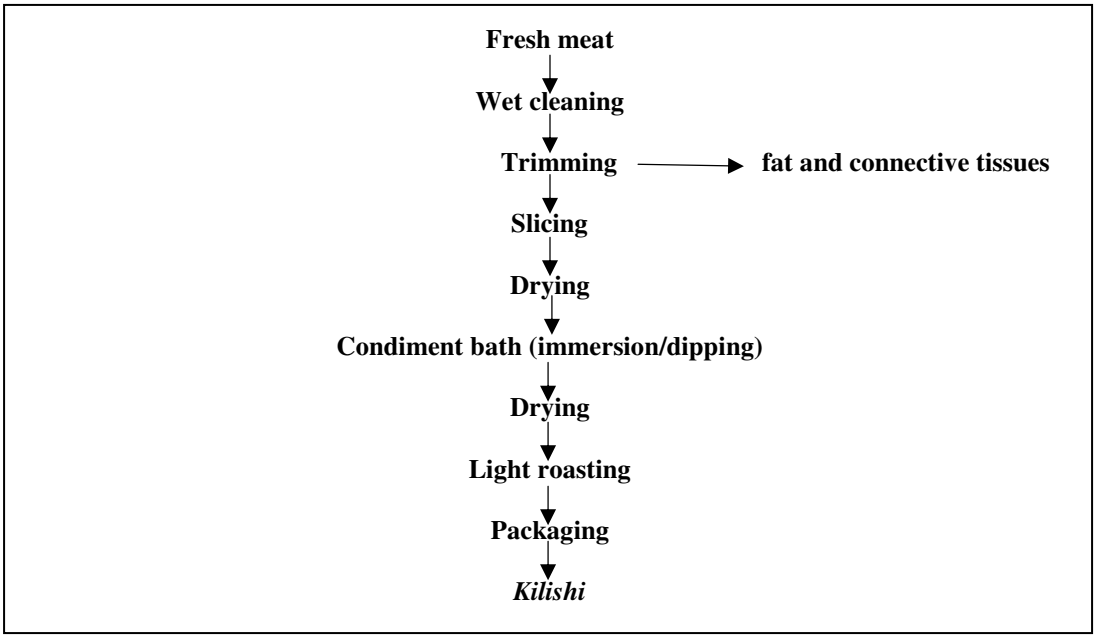

Figure 1: Tradition Kilishi Production Process
Source; Okonkwo et al. (2013)

Sample Code	Sucrose	Citric	Sodium Acid	Condiment Benzoate	Meat
001	0.0	0.0	0.0	45.0	55
014	2.0	0.1	0.1	42.8	55
017	2.0	0.2	0.1	42.7	55
023	4.0	0.1	0.1	40.8	55

Table 2: Percentages of Hurdles and Condiment used in Kilishi

2.4. Microbiological Analyses

Determination of fungal count was performed using acidified potato dextrose agar using serial dilution method described by the American Public Health Association (APHA, 1992). Isolates were identified by morphological characteristics and microscopic examination using procedure described by Chessbrough (2000).

3. Results

Sample Code	Packaging Materials	Storage Time (weeks) 0	2	4	6	8	10	12
		Yeast and Mould Count ($\times 10^2$ cfu/g)						
001	HDPE	1.10	1.18	1.26	1.30	1.36	1.39	1.44
	Aluminium foil	1.10	1.21	1.32	1.40	1.48	1.53	1.60
	Brown paper	1.10	1.28	1.39	1.54	1.66	1.72	1.83
014	HDPE	0.89	0.94	1.01	1.08	1.13	1.19	1.22
	Aluminium foil	0.89	0.98	1.08	1.11	1.17	1.21	1.28
	Brown paper	0.89	0.98	1.10	1.17	1.24	1.29	1.39
017	HDPE	0.86	0.89	0.96	1.00	1.08	1.13	1.19
	Aluminium foil	0.86	0.92	0.99	1.11	1.16	1.20	1.26
	Brown paper	0.86	0.95	1.00	1.19	1.24	1.28	1.36
023	HDPE	0.82	0.88	0.93	0.99	1.04	1.09	1.22
	Aluminium foil	0.82	0.90	0.98	1.03	1.09	1.20	1.28
	Brown paper	0.82	0.94	1.01	1.00	1.19	1.36	1.43
MS	HDPE	3.30	3.46	3.50	3.54	3.62	3.69	3.74
	Aluminium foil	3.30	3.52	3.61	3.67	3.71	3.81	3.90
	Brown paper	3.30	3.60	3.69	3.75	3.81	3.89	3.93

Table 3: Effect of hurdles and Packaging materials on fungal count in Kilishi samples under ambient storage (30 ± 8^0c)

Sample codes		001	014	017	023	MS
				% Hurdles		
	Sucrose	0	2	2	4	0
	Citric acid	0	0.1	0.2	0.1	0
	Sodium benzoate	0	0.1	0.1	0.1	0
Fungal group		**Fungal Distribution (%)**				
Mucor		60	65	60	45	26
Rhizopus stolonifer		20	15	20	25	30
A. Flavus		10	10	10	15	20
A. fumigatus		10	10	10	15	24
Total		100	100	100	100	100

Table 4: Fungal distribution in Kilishi samples treated with different hurdles under ambient storage (30±8^{0}c) (Week 0)

Sample codes		001	014	017	023	MS
				% Hurdles		
	Sucrose	0	2	2	4	0
	Citric acid	0	0.1	0.2	0.1	0
	Sodium benzoate	0	0.1	0.1	0.1	0
Packaging Material	**Fungal group**	**Fungal Distribution (%)**				
HDPE	*Mucor*	60	60	55	45	30
	Rhizopus stolonifer	20	20	25	30	30
	A. Flavus	10	10	05	10	20
	A. fumigatus	10	10	15	15	20
	Total	100	100	100	100	100
Aluminium Foil	*Mucor*	55	65	60	50	30
	Rhizopus stolonifer	20	10	15	25	30
	A. Flavus	10	15	15	20	20
	A. fumigatus	15	10	10	10	20
	Total	100	100	100	100	100
Brown Paper	*Mucor*	60	65	65	50	30
	Rhizopus stolonifer	25	15	15	25	30
	A. Flavus	05	10	10	15	20
	A. fumigatus	10	10	10	10	20
	Total	100	100	100	100	100

Table 5: fungal distribution in Kilishi samples treated with different hurdles under ambient storage (30±8^{0}c) using various packaging materials (Week 2)

Sample codes		001	014	017	023	MS
				% Hurdles		
	Sucrose	0	2	2	4	0
	Citric acid	0	0.1	0.2	0.1	0
	Sodium benzoate	0	0.1	0.1	0.1	0
Packaging Material	**Fungal group**	**Fungal Distribution (%)**				
HDPE	*Mucor*	60	65	55	50	35
	Rhizopus stolonifer	20	15	25	30	30
	A. Flavus	10	10	10	10	15
	A. fumigatus	10	10	10	10	20
	Total	100	100	100	100	100
Aluminium Foil	*Mucor*	60	65	60	50	30
	Rhizopus stolonifer	20	15	20	20	30
	A. Flavus	10	10	15	15	20
	A. fumigatus	10	10	05	15	20
	Total	100	100	100	100	100
Brown Paper	*Mucor*	60	65	60	55	35

	Rhizopus stolonifer	20	10	20	20	30
	A. Flavus	10	10	10	15	15
	A. fumigatus	10	15	10	10	20
	Total	100	100	100	100	100

Table 6: fungal distribution in Kilishi samples treated with different hurdles under ambient storage (30±8^{0}c) using various packaging materials (Week 4)

Sample codes		**001**	**014**	**017**	**023**	**MS**
				%Hurdles		
	Sucrose	0	2	2	4	0
	Citric acid	0	0.1	0.2	0.1	0
	Sodium benzoate	0	0.1	0.1	0.1	0
Packaging Material	**Fungal group**			**Fungal Distribution (%)**		
HDPE	*Mucor*	65	65	60	55	40
	Rhizopus stolonifer	20	20	20	25	30
	A. Flavus	05	05	10	10	15
	A. fumigatus	10	10	10	10	15
	Total	100	100	100	100	100
Aluminium Foil	*Mucor*	65	65	65	55	35
	Rhizopus stolonifer	20	20	25	20	30
	A. Flavus	05	10	05	10	15
	A. fumigatus	10	05	05	15	20
	Total	100	100	100	100	100
Brown Paper	*Mucor*	65	65	60	60	45
	Rhizopus stolonifer	20	15	20	20	30
	A. Flavus	10	10	10	10	10
	A. fumigatus	05	10	10	10	15
	Total	100	100	100	100	100

Table 7: fungal distribution in Kilishi samples treated with different hurdles under ambient storage (30±8^{0}c) using various packaging materials (Week 6)

Sample codes		**001**	**014**	**017**	**023**	**MS**
				%Hurdles		
	Sucrose	0	2	2	4	0
	Citric acid	0	0.1	0.2	0.1	0
	Sodium benzoate	0	0.1	0.1	0.1	0
Packaging Material	**Fungal group**			**Fungal Distribution (%)**		
HDPE	*Mucor*	70	65	60	60	45
	Rhizopus stolonifer	20	25	20	25	35
	A. Flavus	05	05	10	10	10
	A. fumigatus	05	05	10	05	10
	Total	100	100	100	100	100
Aluminium Foil	*Mucor*	70	65	65	60	40
	Rhizopus stolonifer	20	25	25	25	35
	A. Flavus	05	05	05	05	10
	A. Fumigatus	05	05	05	10	15
	Total	100	100	100	100	100
Brown Paper	*Mucor*	70	70	65	60	55
	Rhizopus stolonifer	20	15	25	25	25
	A. Flavus	05	10	10	10	10
	A. fumigatus	05	05	10	05	10
	Total	100	100	100	100	100

Table 8: fungal distribution in Kilishi samples treated with different hurdles under ambient storage (30±8^{0}c) using various packaging materials (Week 8)

Sample codes		001	014	017	023	MS
				% Hurdles		
	Sucrose	0	2	2	4	0
	Citric acid	0	0.1	0.2	0.1	0
	Sodium benzoate	0	0.1	0.1	0.1	0
Packaging Material	**Fungal group**	**Fungal Distribution (%)**				
HDPE	*Mucor*	70	70	65	70	50
	Rhizopus stolonifer	30	25	25	25	30
	A. Flavus	00	00	05	00	10
	A. fumigatus	00	05	05	05	10
	Total	100	100	100	100	100
Aluminium Foil	*Mucor*	70	70	65	65	50
	Rhizopus stolonifer	20	25	25	25	30
	A. Flavus	05	00	05	05	10
	A. fumigatus	05	05	05	05	10
	Total	100	100	100	100	100
Brown Paper	*Mucor*	70	70	70	70	60
	Rhizopus stolonifer	20	20	20	30	25
	A. Flavus	05	05	10	00	10
	A. fumigatus	05	05	00	00	05
	Total	100	100	100	100	100

Table 9: fungal distribution in Kilishi samples treated with different hurdles under ambient storage (30±8^{0}c) using various packaging materials (Week 10)

Sample codes		001	014	017	023	MS
				% Hurdles		
	Sucrose	0	2	2	4	0
	Citric acid	0	0.1	0.2	0.1	0
	Sodium benzoate	0	0.1	0.1	0.1	0
Packaging Material	**Fungal group**	**Fungal Distribution (%)**				
HDPE	*Mucor*	75	70	70	70	50
	Rhizopus stolonifer	25	30	25	30	30
	A. Flavus	00	00	00	00	10
	A. fumigatus	00	00	05	00	10
	Total	100	100	100	100	100
Aluminium Foil	*Mucor*	75	75	70	65	55
	Rhizopus stolonifer	20	25	25	30	25
	A. Flavus	00	00	00	05	10
	A. fumigatus	05	00	05	00	10
	Total	100	100	100	100	100
Brown Paper	*Mucor*	75	70	70	75	65
	Rhizopus stolonifer	25	30	25	25	25
	A. Flavus	00	00	05	00	05
	A. fumigatus	00	00	00	00	05
	Total	100	100	100	100	100

Table 10: fungal distribution in Kilishi samples treated with different hurdles under ambient storage (30±8^{0}c) using various packaging materials (Week 12)

Stated in Table 3 above are the effects of chemical hurdles and packaging materials on fungal counts in *Kilishi* during ambient storage (30±8^{0}C). Sample 001 (control) and Market Sample (MS) contained no chemical hurdle. Samples 014, 017 and 023 were treated with different hurdle combinations as stated above. At the start, the yeast and mould counts for Sample 001, 014, 017 and 023 were found to be 1.10×10^2, 0.89×10^2, 0.86×10^2, 0.82×10^2 and 3.30×10^2 cfu/g respectively. Highest counts were recorded in commercial sample throughout the storage time and least counts were recorded in Sample 017 and 023. Among the packaging materials used HDPE was

found to be more efficient with low counts in all the treatments. Hurdle pattern in Sample 017 with combination of HDPE was found to be the best treatment in retaining the fungal quality of *Kilishi*.

Table 4 to 10 above presented the percentage distribution of fungal groups in *Kilishi* treated with sucrose, citric acid and sodium benzoate at different levels and combinations under ambient storage (30 ± 8^0C) for twelve weeks. Four mould groups were isolated from *Kilishi* samples, these include; *Mucor, Rhizopus stolonifer, A. Flavus* and *A. fumigatus.* In the freshly prepared laboratory samples, *Mucor* was found to have highest percentage distribution ranging from 45 to 65%, least percentages were recorded in *Aspergillus species* which was ranged from 10 to 15%. The mould distribution in the freshly prepared market sample was fairly even and found in the range of 24 to 30%.

Succession in the mould distribution was observed during twelve weeks' ambient storage (30 ± 8^0C). *Mucor* and *Rhizopus stolonifer* with highest initial percentages were found to dominate and eliminate the growth of *A. Flavus* and *A. fumigatus* during the storage time. The distributions of *Mucor* increased from 45% to 75% depending on the hurdle combinations and packaging materials, and that of *Rhizopus stolonifer* were increased from 10 to 25%.

The hurdles combination and packaging materials used in this research were found to have less effect on the growth and distribution of *Mucor, Rhizopus stolonifer, A. Flavus* and *A. fumigatus* during ambient storage (30 ± 8^0C) of *Kilishi.*

A graphical representations of the fungal succession in different packaging materials were presented in Fig 2 to 4 below.

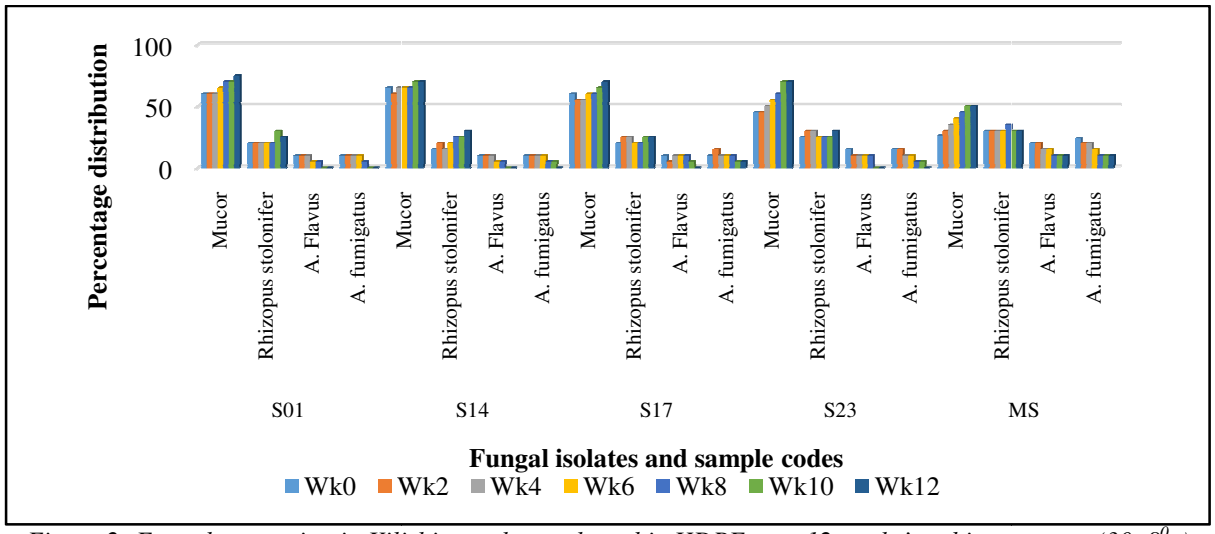

Figure 2: Fungal succession in Kilishi samples packaged in HDPE over 12 weeks' ambient storage (30 ± 8^0c)

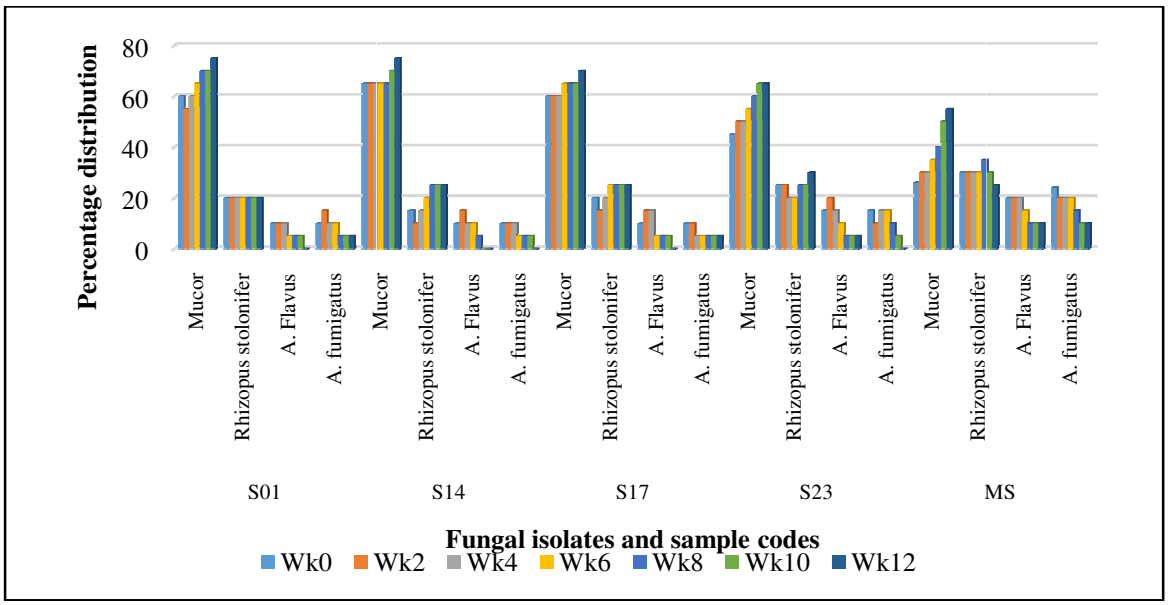

Figure 3: Fungal succession in Kilishi samples packaged in Aluminium foil over twelve weeks' ambient storage (30 ± 8^0c)

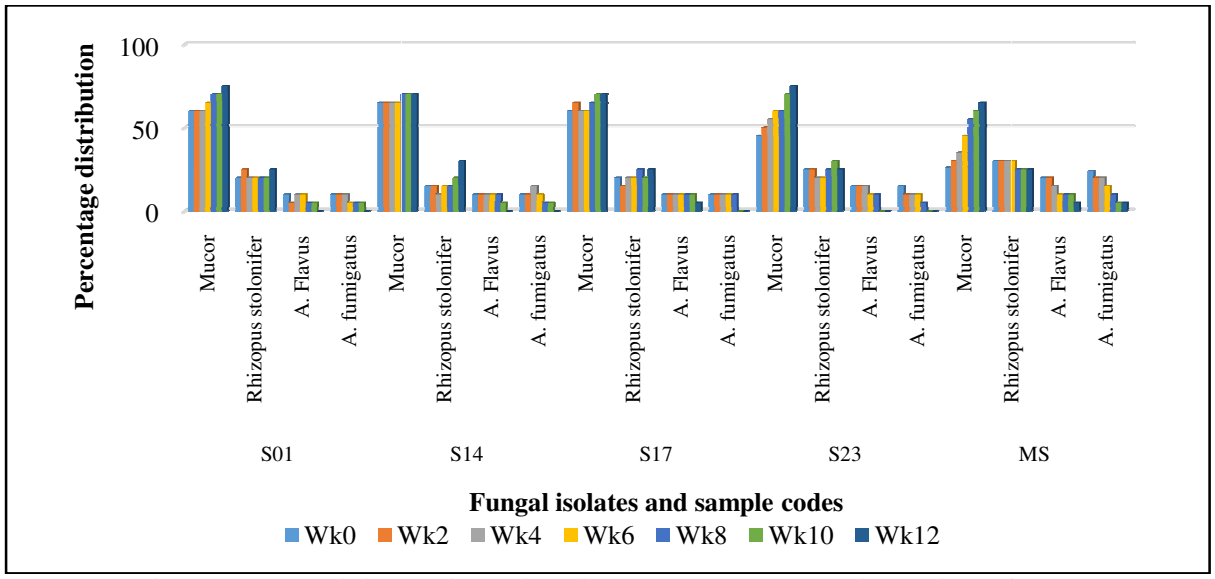

Figure 4: Fungal succession in Kilishi samples packaged in Brown paper over twelve weeks' ambient storage (30±8^{0}c)

4. Discussion

The fungal loads of *Kilishi* were found to be increasing in both commercial and laboratory prepared samples during ambient storage (30±8^{0}C). The fungal loads were increased from 1.10×10^2 cfu/g to 1.83×10^2 cfu/g in sample 001, 0.89×10^2 cfu/g to 1.39×10^2 cfu/g in sample 014, 0.86×10^2 cfu/g to 1.136×10^2 cfu/g in sample 017, 0.82×10^2 cfu/g to 1.43×10^2 cfu/g in sample 023 and 3.30×10^2 cfu/g to 3.93×10^2 cfu/g in market sample. Ogbonnaya and Linus (2009) reported increase in fungal counts during ambient (30±7^{0}C) storage of *Kilishi* treated with potassium sorbet. Increase in fungal counts during storage of *Kilishi* contradict the report of Bibek (2005) who opined that when growth requirements of organisms were altered by addition of preservatives, their growth in foods can be reduced drastically for a considerable period of time.

The results for fungal count in freshly prepared control sample was found to be below that reported by Ogbonnaya and Linus (2009). The fungal count in market sample collected from Kano was found to be below that reported by Egbebi and Seidu (2011) in commercial *Kilishi* samples collected from Ado-Ekiti and Akure in Ekiti and Ondo States respectively. Fungal counts for laboratory prepared *Kilishi* were found to be within the range reported by Ogbonnaya and Linus (2009)

Bibek (2005) opined that when growth parameters were tackled by using many preservatives the growth will either stop or occur at a very slow rate, or death may even during storage. Jones *et al.* (2001) reported that treatment of*Kilishi* with potassium sorbet and polythene packaging will confer a degree of product protection from mould contamination.

Mucor, Rhizopus stolonifer, A. Flavus and A. fumigatus were identified in all the samples. The growth pattern observed was very similar to that reported by Jones *et al.* (2001). Microbiological succession was observed during storage in the laboratory prepared samples where *Mucor* and *Rhizopus stolonifer* subsided the growth of *A. Flavus* and *A. fumigatus* before the end of the storage period. This is in agreement with the finding of James (2000) who reported *Mucor* and *Rhizopus* as the predominant spoilage fungi of meat. Stanojevic*et al* (2009) reported that *Aspergillus flavus* showed greater resistance than other fungal species in a growth media treated with sodium benzoate, sodium nitrite and potassium sorbet. Heydaryinia*et al.* (2011) reported that 0.1% sodium benzoate can inhibit the growth of *A. niger* in yeast extract sucrose broth, contained 2% yeast extract and 15% sucrose. It should be noted that the microbial profile of food is quite different from that of a pure culture growing in a laboratory medium (Bibek, 2005)

Many researchers reported similar fungal groups during ambient storage of *Kilishi*; Faleye andFagbohun (2012) during 18 weeks' storage of dried meat sold in Lagos State-Nigeria isolated *Rhizopus nigricans, Mucor spp*., *Aspergillus flavus, Aspergillus niger, Fusariummonoliforme, Absidiacandidus, Aspergillus glaucus*and *Penicilliumspp*. Similar isolates were also reported by Egbebi and Seidu (2011) in *Kilishi* samples collected from Ado-Ekiti and Akure in Ekiti and Ondo State respectively. Contrary to this research, Egbebi and Seidu (2011) reported that *Aspergillus spp* were found to have maximum percentage of occurrence among fungal species in *Kilishi.*

James (2000) reported that microorganisms differ in their response to hypertonic concentrations of sugars, with yeasts and molds being less susceptible than bacteria. Some yeasts and molds can grow in the presence of as much as 60% sucrose, whereas most bacteria are inhibited by much lower levels (James, 2000). Piper *et al.* (1997) cited in Brul andCoote (1999) reported that fungi are more resistance to organic acid preservatives when compare with bacteria. He also reported that the long term stress response of yeasts to weak organic acids also involves the outer induction of an integral membrane protein.

5. Conclusion

The results of the study showed that combination of sucrose, citric acid and sodium benzoate can improve traditional method of *Kilishi* production. Increase in fungal count was observed during ambient storage (30±8^{0}C). Highest counts were recorded in market sample and least counts were recorded in *Kilishi* sample treated with 4% sucrose, 0.1% citric acid and 0.1% sodium benzoate. The results of the research revealed that packaging *Kilishi* in HDPE provides better microbial quality than aluminium foil and brown paper

during ambient storage. *Mucor, Rhizopus stolonifer, Aspergillus fumigatus* and *Aspergillus flavus* were found to be presence in all the samples. *Mucor, Rhizopus stolonifer* took over the growth of *Aspergillus fumigatus* and *Aspergillus Flavus* in all the laboratory prepared samples before the end of the storage time. Incorporation of 4% sucrose, 0.1% citric acid and 0.1% sodium benzoate and use of HDPE as packaging material in tradition production of *Kilishi* will provide product with improved microbial quality.

6. References

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