SURVIVAL OF SELECTED PATHOGENS DURING THE PRODUCTION AND STORAGE OF *IRU* A FERMENTED VEGETABLE CONDIMENT

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ABSTRACT
Pathogens have been isolated from some fermented foods indicating their capability of growing and surviving the fermentation process, indicating that there could be a food safety risk if these pathogens survived in fermented foods. Therefore, the need to investigate the survival of some bacterial pathogens during the fermentation and storage of iru a traditional fermented condiment from African locust beans (Parkia biglobosa). Pathogens used were Pseudomonas aeruginosa, Salmonella Typhi, Staphylococcus aureus and Escherichia coli. They were inoculated into fermenting iru and during storage at salt concentrations (2.5, 5, 7.5, and 10%) and temperatures (4, 25, 37 and 42 ° C) for 5 days. Salmonella Typhi was inhibited after 48 h, E. coli after 72 h, P. aeruginosa after 24 h while S. aureus survived until the last day during iru fermentation. Most of the pathogens survived between salt concentrations of 2.5 % and 7.5 % but their growths were inhibited at 10 %. Only S. aureus survived all the salt concentrations during storage. Temperature of 42 °C inhibited the growth of S. Typhi and E. coli over a storage period of 5 days while P. aeruginosa was inhibited at 4 °C. Inhibition of growth of pathogens during fermentation might be due to the bacteriostatic and bactericidal properties of the indigenous organisms, more so, the fermentation process is an alkaline fermentation, which makes it difficult for the growth of the pathogens introduced. Storage of iru using 10 % salt concentration could be a safe way of storing the condiment and proper hygiene condition is recommended to eradicate S. aureus.

Keywords: Iru; pathogens; production; storage; survival rate

Received: 15.06.2018 Reviewed: 10.09.2018 Accepted: 28.09.2018

1. INTRODUCTION

Fermented foods are essential parts of diets in all regions of the world, particularly in Africa and Asia. They are produced from different substrates or raw materials, which include vegetables. Leguminous seeds account for about 80% of the dietary protein, which are usually used in fermented form as condiments to enhance the flavours of foods and soups (Odunfa, 1981; Achi, 2005). Some of the most important food condiments are 'iru' or 'dawadawa' from African locust bean (*Parkia biglobosa*) and 'Ogiri' (*Citrullus vulgaris*) (Odunfa, 1985a; Ogunshe *et al*., 2006). Due to the high cost of animal proteins, protein - rich oily seeds such as African locust beans are fermented as sources of proteins. They are consumed in developing countries where consumption of animal proteins may be limited as a result of economic, social, cultural or religious factors (Odunfa and Adewuyi, 1985; Sanni and Ogbonna, 1991; Esenwah *et al*., 2008). Indigenous microorganisms, thus, carry out the process of fermentation of most fermented foods in Africa leading to some undesirable effect on the nutritional, quality and safety of the final product. This process of natural fermentation also signifies that the possibility of the involvements of pathogenic microorganisms and spoilage organisms during the production process cannot be ruled out. Pathogens have been isolated from some fermented foods, indicating that they are capable of growing in the food or surviving the fermentation process (Gadaga *et al*., 2004; Neeraj and Sharma, 2007). In South Africa, Kunene *et al*. (1999) reported that 40% of fermented sorghum meal samples contained *Bacillus cereus* while 8% contained *Escherichia coli*. Olasupo *et al*. (2002) reported that among microorganisms of public health concern, *Staphylococcus aureus* and *Klebsiella* sp. were isolated from 'wara' a fermented cheese-like product, while *E. coli, Salmonella*

sp. and *Klebsiella* sp. were isolated from 'nono', 'ogi' and 'kunu – zaki'. Contamination of foods by pathogenic organisms remains one of the major public health problems worldwide and a wide variety of diseases occurs by eating foods contaminated with pathogenic microorganisms. Examples of such diseases include cholera, salmonellosis, hepatitis A, acute aflatoxicosis or entero – haemorrhagic *Escherichia coli* (EHEC) among several others (FAO, 2005). The most widely encountered pathogens in many African fermented foods are *E. coli, Pseudomonas aeruginosa, Klebsiella* sp., *Salmonella* sp., *Vibrio cholera, Shigella* sp., *Campylobacter, Staphylococcus aureus* (Gadaga *et al*., 2004). The ability of pathogens to grow in fermented foods, which may result in foodborne diseases, involves the microorganisms overcoming some hurdles like pH changes, low water activity, low redox potential and in some cases, heat treatments and natural antimicrobial compounds. Neeraj and Sharma (2007) reported that the survival of microorganisms, in general, is dependent on a variety of factors (which could be intrinsic or extrinsic) that determine whether the microorganisms will preserve or spoil the food. In West Africa, "iru" is one of the most important food condiments available. It is normally obtained by the traditional alkaline fermentation of African locust bean (*Parkia biglobosa*). Traditionally, its production involves boiling of the seeds for 24–40 hours, dehulling, second boiling for 1–2 hours, fermentation for 24–72 hours (25–37 °C). There are two major types depending on the length of fermentation time. *Bacillus* spp. has been reported as the main microorganisms responsible for the fermentation of African locust bean seeds to "iru". The most predominant species is *Bacillus subtilis*, but other species such as *B. pumilus, B. licheniformis, B. megaterium, B. cereus, Staphylococcus* spp, *Micrococcus* spp have also been identified (Ouaba *et al*., 2010). There is no previous information in Nigeria on how these pathogenic microorganisms survived during the production and storage of *'iru'* and there is very little information on the occurrence and growth of pathogens in African fermented foods generally. Therefore there is a need to study the survival rate of these pathogens during the production and storage of *'iru'* so as to assist in the development of a hazard analysis and critical control points (HACCP) plan for the production of a safe *'iru'* to consumers at large.

2. MATERIAL AND METHODS

Sample Collection:

Fresh African locust bean seeds (*Parkia biglobosa*) used for this study was purchased at Bodija market, in Ibadan metropolis, Oyo state, Southwest Nigeria.

Sources of Pathogens:

The following pathogens *Escherichia coli*, *Salmonella* Typhi, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from the culture collection centre of the Department of Medical Microbiology of the University College Hospital (UCH), Ibadan, Nigeria. The pathogens were sub-cultured in Tryptic Soy Broth (TSB) (incubated at 30 °C for 24 h) and maintained on Tryptic Soy Agar (TSA) (Becton Dickinson AG) slant at 4 °C.

Determination of inoculum size:

The inoculum size used was determined using the method of Omafuvbe *et al*. (2006). Breifly, the pathogens were grown on Nutrient agar (Thermo Fisher Diagnostics AG) at 37 °C for 18 – 24 h. Each of the isolates were harvested and suspended into 10 ml sterile peptone water (Thermo Fischer Diagnostics AG) and serially diluted to give an absorbance of 0.03 at 540 nm using a UV/VIS spectrophotometer (Jenway 5210, Essex, United Kingdom). A volume of 0.3ml of predominantly vegetative cells from the various dilutions of pathogens was inoculated into 30g of cooked African locust bean seeds in a sterile Petri dish.

Estimation of pathogens:

Culture media used were Mannitol Salt Agar (12CNS GmbH), Centrimide agar (VWR International AG), Bismuth Sulphite agar (SIGMA- ALDRICH, SWIT) and Eosin Methylene Blue agar (Becton Dickinson AG). For enumeration of the pathogens, 1g of the

fermented *iru* was taken aseptically at 0 h and at every 24 h for a period of 4 days using a sterile spatula and transferred into a sterile stomacher bag , mix thoroughly using a Stomacher blender (Huber & Co. AG) containing 9 ml of peptone water. Pour plate technique was employed using the appropriate media as applicable. Incubation of the plates was done at 30 °C for 24 – 48 h after which the colonies were counted.

Determination of Temperature and pH:

The temperature was determined by inserting a sterile thermometer into the fermenting sample at every 24 h interval for 4 days. pH was determined by taking 5 g of the sample at every 24 h and mashed in a sterile mortar and pestle after which it was transferred to a clean beaker and 100 ml of distilled water was added. This was mixed thoroughly to make a slurry, allowed to settle, then decanted and the pH of the filtrate was measured using the H^+ electrode of a digital pH meter (Mettler Toledo, Fisher Scientific, Massachusetts, USA). The readings were taken in triplicates and the mean calculated.

Effect of temperatures on the survival of pathogens during storage of *iru*:

Thirty grammes of *iru* was inoculated with the different pathogens and stored at temperatures 4 °C, 25 °C, 37 °C and 42 °C respectively. At 24 h interval, 1g of the sample stored at the different temperature was taken and the pour plate technique was used to determine the survival of the pathogen at the storage temperatures.

Effect of NaCl on the survival of pathogens during storage of *iru*:

Fermented African locust beans *iru* was transferred into sterile containers with varying concentrations of NaCl (0 %, 2.5 %, 5 % and 10 %). The pathogens were inoculated into the samples and kept at ambient temperature (28 ºC). At 24-h interval, one gramme was taken from each sample to determine the rate of survival of the pathogens.

3. RESULTS

Survival rate of pathogens during *iru* fermentation:

During the fermentation process of *iru*, the population of *Escherichia coli* decreased from its initial load of 5.50 CFU/g at 0 h to 4.20 CFU/g after 72 h and ceased from surviving at 96 h. There was an increase in the pH of the fermented product from 8.22 at 0 h to 8.98 at 96 h and the fermentation temperature increased from 24 °C to 39.5 °C. The population of *Salmonella* Typhi increased at 24 h in the fermenting *iru* but reduced after 48 h and ceased to survive after 72 h. *Pseudomonas aeruginosa* was not detected in the fermenting African locust beans after 48 h. The pathogen only survived for 24 h of exposure with an increase in pH and temperature of the fermenting seeds. The rate of survival of *Staphylococcus aureus* during the fermentation of *iru* was uniform all through the period of fermentation. There was an initial decrease in the population size from 5.91 log CFU/g to 5.72 log CFU/g after 24 h. Thereafter, it increased at 48 h to 6.00 log CFU/g and later reduced again from 72 to 96 h. The fermentation process generally showed an increase in pH and temperature. Results are shown in Table 1.

Effect of salt concentration on pathogens during storage:

The effect of the different salt concentrations on the survival of the pathogens is presented in Figure 1. *Escherichia coli* survived the 5 days storage period of *iru* with a reduced growth rate at higher salt concentration while the growth was totally inhibited at day 3 in 10 % salt concentration. There was a significant reduction in the growth of *E. coli* as the salt concentration increased from 2.5 % to 10 %. For *P. aeruginosa*, there was a significant reduction in the survival rate after 2 days of storage. At day 3 and 4, there was an increase in growth. At day 3, the microbial load was 6.11 log CFU/g, 6.0 log CFU/g, 5.78 log CFU/g and 5.7 log CFU/g in 2.5 %, 5 %, 7.5 % and 10 % salt concentration respectively. The microbial count increased at day 4 while at day 5, the growth reduced for all the salt concentrations. The growth of *P. aeruginosa* was well favoured at the 2.5 % salt concentration. *Salmonella* Typhi survived at

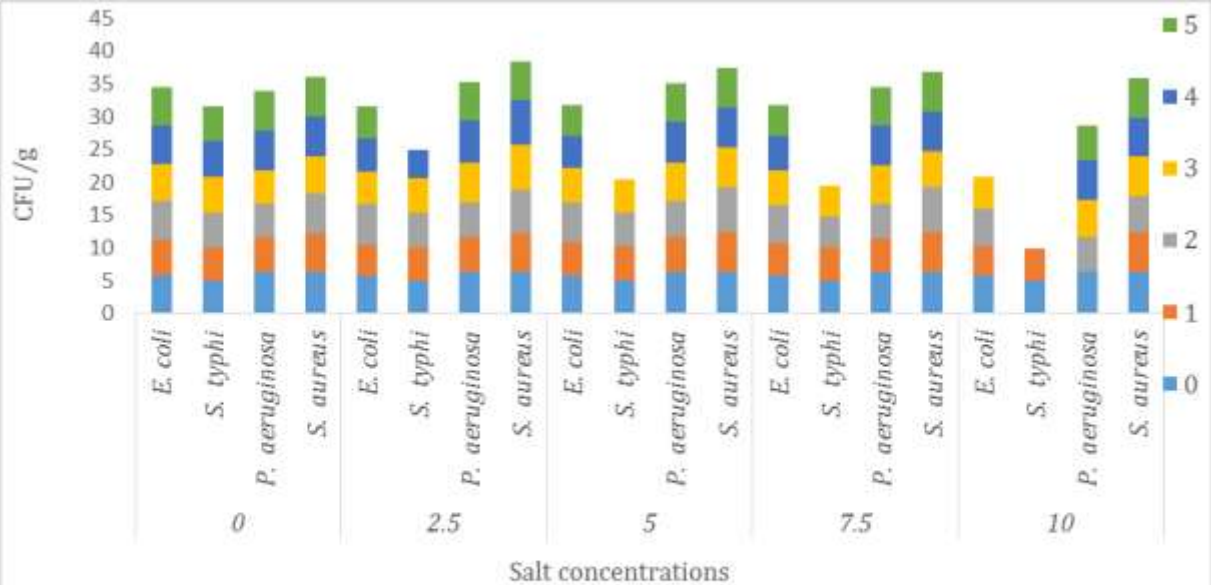

Figure 1: Effect of salt concentration on the survival of pathogens in fermented iru
E. coli: *Escherichia coli*; S. Typhi: *Salmonella* Typhi; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S.aureus*: *Staphylococcus aureus*

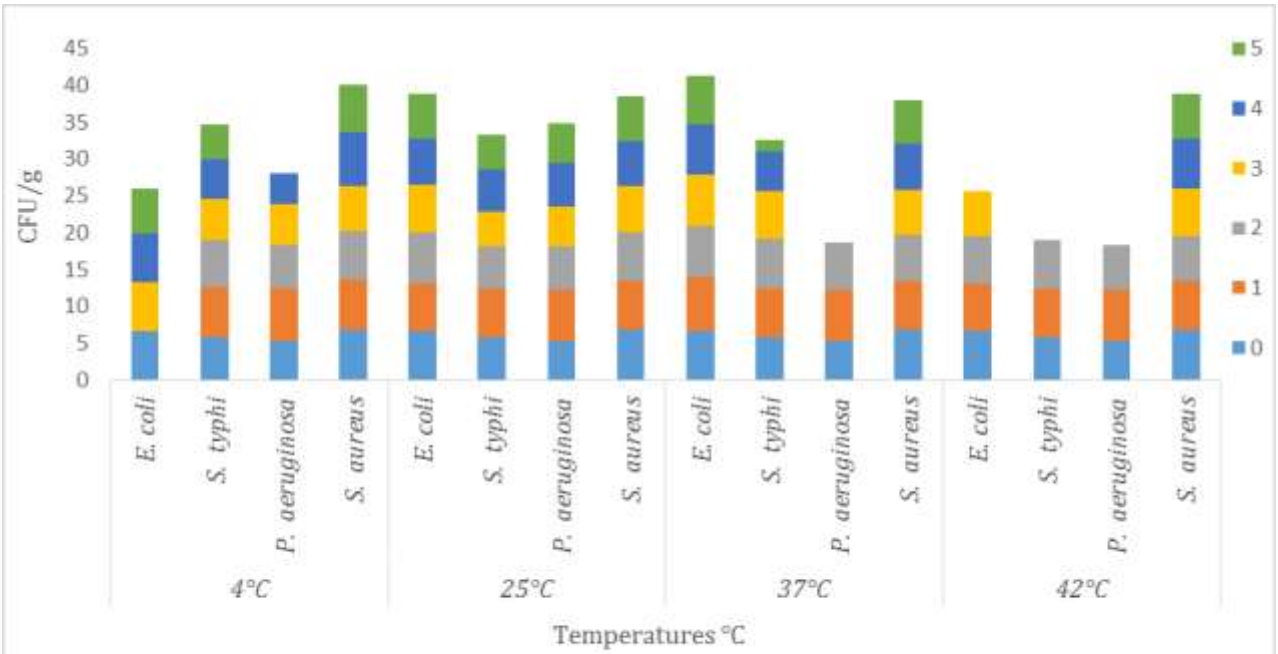

Figure 2: Effect of temperature on survival of pathogens in *iru*
E. coli: *Escherichia coli;* S. Typhi: *Salmonella* Typhi; P. aeruginosa: *Pseudomonas aeruginosa*; S. aureus: *Staphylococcus aureus*

2.5 %, 5 % and 7.5 % salt concentration from day 1 to 3. At day 4, it survived in *iru* with 0% and 2.5% salt, but did not survive at day 5. At 10 % salt concentration, *Salmonella* Typhi was not detected in *iru* after day 1.

Effect of temperature on pathogens during storage:

The effect of different temperatures on the survival of selected pathogens is shown in Figure 2. During storage of *iru*, *E. coli* survived at 37 °C, while at 42 °C there was a reduction in the number of colonies as the days progressed. *E. coli* did not survive during

storage of *iru* at 42 °C after 4 to 5 days. The growth and survival of *E. coli* was favoured best at 25 °C over a period of 5 days. *P. aeruginosa* survived at day 1 in *iru* stored at all the different temperatures, however, the number of colonies reduced at day 2 for all temperature and at day 3, 4 and 5 it did not survive at 37 °C and 42 °C. For *Salmonella* Typhi, it was observed that the pathogen survived at all the temperatures at day 1 and 2. However, there was no growth at 42 °C from days 3 to 5. *Staphylococcus aureus* survived in *iru* at the different temperatures throughout the 5 days of storage, although there was a gradual decrease in the growth until the third day. Highest survival was on day 4 at 4 °C with 7.30 CFU/g. However, 37 °C significantly inhibited the growth of *S. aureus* when compared to the growth at the other temperature.

3. DISCUSSION

Previous studies by Odunfa (1985b) and Antai and Ibrahim (1986) shows that *Bacillus subtilis*, *Leuconostoc mesenteroides* and *Staphylococcus* species were isolated during the fermentation of the seeds of *Parkia biglobosa*, the authors established that *B. subtilis* is the dominant agent of fermentation of African locust beans. Several researchers have isolated *Staphylococcus aureus* from *iru*, but they are assumed not to play any important role in the fermentation process of *iru*. This organism has been classified as ubiquitous during the early stage of the fermentation process with the population increasing rapidly within 24 – 48 h of fermentation and then decreases as fermentation progresses (Olasupo et al., 2002).

From health point of view, the presence of *S. aureus* can be linked to poor hygienic practices and this organism has the potential to produce diarrhoeagenic toxin. The *B. subtilis*, which is the predominant organism, are proteolytic in nature and have the capacity to hydrolyse the locust bean protein to peptides and amino acids. This hydrolysis leads to the release of ammonia, thus causing a rapid increase in pH. Combination of the high pH and free ammonia produce, along with the rapid growth of the essential microorganisms at relatively warm temperature above 40 °C makes it very difficult for other microorganisms especially the pathogens that might spoil the product to thrive (Steinkraus, 2002). Members of the Enterobacteriaceae are usually present at the beginning of the fermentation when the pH is low but disappear at the later stage because of the increase in pH thereby leading to succession by other microorganisms suited for an alkaline environment (Odunfa, 1985a). Achi (2005) reported that members of the Enterobacteriaceae do not survive until the end of the fermenting vegetable condiments presumably because of the increase in pH in the range of 8 to 10, which had developed at later stages of the process. The increase in pH is due to proteolysis that leads to the release of amines and amino acids from the leguminous seed. This explains the reason why *E. coli, S.* Typhi *and P. aeruginosa* could not survive beyond 72 h and 48 h respectively in the fermenting African locust bean, because the fermentation of African locust bean seeds tend the pH toward alkalinity, pH 8 and above (Odunfa, 1985a; Sanni and Oguntoyinbo, 2014). The low initial pH of the fermentation process makes it possible for some organisms that are of public health concern to survive at the initial fermentation period, which is risky to the community because *iru* can be eaten raw without pre heating. Post - processing techniques proposed for condiment production and preservation in Africa include drying and salting of the product (Achi, 2005). The sodium ions associate with water molecules to reduce the amount of unbound water in foods, making it difficult for the microorganisms to grow (Henry and Taylor, 2010). Salt has the ability to stimulate osmotic shock in microbial cells, thereby, affecting the growth of the microbe and promoting its cell death (Davidson et al., 2013). Several types of researches have shown that the permeability of the *S.* Typhimurium cells are easily altered by heat and this allows the sodium ions to penetrate the cell into the cytoplasm and interfere with the

cell metabolism (Manas et al., 2001). Although there are, limited studies investigating the effect of salt concentration on *Salmonella* spp. there have been studies demonstrating that these microorganisms are capable of enduring extended starvation and desiccation stresses (Finn et al., 2013). The high virulence of this pathogen combined with its high environmental persistence, make *Salmonella* spp. a major challenge to the food industry (Humphrey, 2004). Exposure of *Salmonella* spp. to osmotic or desiccation stress results in the loss of water that causes considerable shrinkage of the microbial cells (Csonka, 1989) with a consequent increase in concentrations of all the intracellular metabolites. Sudden plasmolysis may result in the inhibition of a variety of physiological processes, ranging from nutrient uptake to DNA replication (Csonka, 1989; Starzzullo et al., 2009).

The effect of different salt concentrations on *S. aureus* during the storage of *iru* does not have any significant difference on the growth of the pathogen. *Staphylococcus aureus* survived all the different salt concentrations from day 1 to 5. Tolerance of *S. aureus* to high concentrations of NaCl has been reported with no damage shrinkage observed in its cellular structure (Omotoyinbo and Omotoyinbo, 2016). *E. coli* can grow without the presence of salt but can also tolerate little amount of salt in a growth medium. Even though *E. coli* seems to have optimal growth in the absence of salt, the organism grows at a slow rate in the presence of salt (Ryan, 2017). However, the higher the NaCl concentration, the lower the bacteria reach its optimal growth. Hajmeer (2001) and Abdulkarim *et al*. (2009) reported the decrease in growth rate in *E. coli* and *S. aureus* at higher salt concentrations. It was suggested that this might be due to the hyper osmotic shock effect on the organism thereby leading to growth suppression. The decrease in population with increasing concentration of NaCl can be attributed to the exposure of the organisms to conditions of hyper osmolarity resulting in a decrease in their cytoplasmic water activities. NaCl increases the osmolarity of the medium, which in turn causes the loss of the intracellular water with a concomitant increase in the osmolarity of the intra cellular contents (Botsford, 1984; Abdulkarim et al., 2009). The major effect is that proteins or enzymes and other biological macromolecules have evolved to function only within certain normal ranges of water activities; outside which some essential cellular functions become impaired (Csonka, 1989). The range of temperature (7 – 46°C) for the growth of Salmonella as reported by ICMSF, (1980), indicates that at 4°C growth would not be possible. The study revealed that at 4°C the growth of Salmonella sp. was low. However, factors such as the water activity of the samples as well as other biological factor might have contributed to the survival of the Salmonella sp. at the storage temperature of 4°C. This implies that at 4°C the microbe exert more effort to survive by adjusting to that condition hence the lower growth rate experienced.

4. CONCLUSION

It is imperative for fermented foods to have a very good safety record, irrespective of the preparation environments. However, the survival of pathogens in fermented foods, as observed in this study to some certain extent,

TABLE 1. Microbial load of pathogens, pH and temperature change during iru fermentation

	Microbial load (Log cfu/g)				pH				Temperature (°C)			
Hours	*Esch. coli*	*Salm.* Typhi	*Pseud. aeruginosa*	*Staph. aureus*	*Esch. coli*	*Salm.* Typhi	*Pseud. aeruginosa*	*Staph. aureus*	*Esch. coli*	*Salm.* Typhi	*Pseud. aeruginosa*	*Staph. Aureus*
0	5.50	5.50	6.20	5.91	8.22	8.35	8.35	8.22	24	23	25	24
24	5.30	5.60	5.40	5.72	8.26	8.36	8.43	8.35	27	27	28.5	27
48	5.00	4.60	0.00	6.00	8.61	8.54	8.70	8.68	30.5	28	32.0	30.5
72	4.20	0.00	0.00	5.90	8.54	8.82	8.90	8.72	34.5	35	36.0	34
96	0.00	0.00	0.00	5.45	8.98	8.93	8.94	9.00	39.5	38	39.0	38.5

suggests that measures that will help to minimise the risk of foodborne illnesses need to be taken. Application of HACCP should be functional to a wider range of fermented foods production processes and education of the processors is paramount to the implementation of the development.

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