



Modelling the effects of local food spices on the growth of *Salmonella typhimurium*.

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Abstract

Meat and coleslaw are frequently exposed to food contaminants from handling and during processing. Biological, chemical and physical agents have been used to inhibit food contaminants. The activity of food spices (*Ocimum gratissimum*, *Piper guineense* and salt) were investigated for their ability to inhibit or stimulates the growth of *Salmonella typhimurium* isolated from meat and coleslaw samples in singles and in combination. The isolates were subjected to temperature dependent inactivation for the development of primary models using the GinaFit Software. A 2^k. Factorial plot was carried out to determine the single and combined effect of the spices on the test isolates. Results shows that at a concentration of 800 µg/ml *Ocimum gratissimum* extracts had stationary/inhibitory effect, *Piper guineense* also had stationary/inhibitory effects while salt had inhibitory effects at 4% concentration. At 8% concentration, results indicated a bactericidal effect as the mean growth declined rapidly below the initial bacterial population. A similar result was also recorded for *Ocimum gratissimum* at 1200 µg/ml. Results on *Salmonella typhimurium* shows that the *Piper guineense* extracts had mean stimulatory effect at 400 µg/ml, while the salt extracts had similar results at 4% salt concentration. Mean inhibitory effect was obtained for *Ocimum gratissimum* extracts at 400 µg/ml and 800 µg/ml but, stimulatory at 1200 µg/ml. At a concentration of 800 µg/ml, *Piper guineense* extracts had inhibitory effects, while salt solution also had stimulatory effects at 8% concentration. At 12% salt concentration, the effect on mean growth was bactericidal demonstrated by rapid decline below the initial bacterial population. GINAFIT Models were used to determine values of “ μ , λ , $\log N$, k_{max} , R^2 , MSE” which were adopted for model development. Findings had it that at -4 and 4 degrees, the isolates was inactivated. In addition, *Salmonella typhimurium* was activated at 25, 35 and 45 degrees but inactivated at -4 degrees. The study strong recommends the use of spices and brine solution in enhancing the shelf life of meat and coleslaws.

Keywords: ABIS Online, *Salmonella typhimurium*, Models, Meat, Vegetables.

Introduction

The use of plants as sources of remedy to different ailments, food and shelter is dated far back to 1979. Among others, the burden of food-borne infections and illnesses is globally ravaging. Africa has numerous food spices of plant origin with promising features, and diverse applications (Okorundu *et al.*, 2015) Many of these plant spices have been explored for antimicrobial properties (Adeleye *et al.*, 2018).

However, fewer of the plants have been modeled for industrial applications. Scent leaf (*Ocimum gratissimum*), Uziza leaf (*Piper guineense*) and Salt are common spices used in Nigerian delicacies which have also found a wide application in medicine. Scent leaf also known as *Ocimum gratissimum* is an herbaceous plant which belongs to the Labiatae family. It is an important plant with several medicinal, ethnomedicinal and nutritional values. Among other potentials; *Ocimum gratissimum* has been used

extensively in the traditional system of medicine in many countries. The flowers and the leaves of this plant are rich in essential oils so it is used in preparation of teas and infusion (Rabelo *et al.*, 2003). In the coastal areas of Nigeria, the plant is used in the treatment of epilepsy, high fever and diarrhea (Effraim *et al.*, 2003). In the Savannah areas decoctions of the leaves are used to treat mental illness (Akinmoladun *et al.*, 2007). *Ocimum gratissimum* is used by the Ibos of Southeastern Nigeria in the management of the baby's cord, to keep the wound surfaces sterile. It is also used in the treatment of fungal infections, fever, cold and catarrh (Ijeh *et al.*, 2005).

Piper guineense is a spice plant from the family *piperaceae* and from genus *piper*. It is a West African spice plant commonly called ashanti pepper (Okoye and Ebeledike, 2013). It is known as *Uziza* in Igbo and *Iyere* in Yoruba. Other common names are benin pepper, guinea pepper, false cubeb and Kale (Tapsel *et al.*, 2006). *Piper guineense* have nutritional and non-nutritional factors which are responsible for its aroma, flavour and preservative properties and proximate analysis of the plant shows that it contains crude protein, fat carbohydrates and vitamins (Nwankwo *et al.*, 2014). Okonkwo and Ogu (2014) reported that the plant contains vitamin C in considerable amount and this could aid the good health of teeth and gums and also promote healing.

According to Anyanwu and Nwosu (2014), *Piper guineense* by its nature is aromatic and carminative and that it is a natural antioxidant, act as anti-inflammatory, anti-cancer and anti-pyretic agents.

Meat and vegetables are good media for the growth and proliferation of pathogenic microorganisms (Chai *et al.*, 2017; Braide *et al.*, 2017). This is because they contain most of the vital minerals, organic carbon and nitrogen sources and water activity. Microorganisms, ambient or transient, find these food materials interesting owing to the fact that their properties support microbial proliferation. As a result, the need to inhibit the microbial activity by food processing industries that use them as raw materials is highly needed (Chai *et al.*, 2017).

This burden and cost of food preservation, and the increased rate of incidence of food-borne infections, locally set a challenge to food microbiologist and places the need for methods that could substitute conventional food processing preservatives and preservation methods. This drives the research into the

use of locally available spices to inactivate microbial activities in a procedure known as "Predictive Microbiology". In the first book on the subject, published just over 20 years ago, McMeekin *et al.*(1993) defined it as a quantitative science that enables users to evaluate objectively the effect of processing, distribution and storage operations on the microbiological safety and quality of foods. The goal of Predictive Microbiology is to develop mathematical equations that describe the behaviour of microorganisms under different environmental factors. The term *Salmonella* refers to a group of bacteria that cause *Salmonella* infection, or *Salmonellosis*, in the intestinal tract. *Salmonella* are gram-negative, rod-shaped bacilli that can cause *Salmonellosis*, a diarrheal illness in humans. It is a Gram-negative bacterium that usually has a cell wall composed of a thin layer of peptidoglycan, covered by a membrane. There are over 2,300 subtypes of the *Salmonella enterica* bacterium, including *Serovars enteritidis*, *Salmonella Agbeni*, and *typhimurium*. The bacteria live in the gut of infected humans and animals. Some animal and human strains can make humans sick. *Salmonella* is a major cause of human bacterial infections in the world. According to the Centers for Disease Control and Prevention (CDC), it affects thousands of people every year, leading to 19,000 hospitalizations and 380 deaths. *Salmonella* poisoning is often linked to contaminated water or foods, especially meat, poultry, and eggs. Symptoms include abdominal cramps and vomiting, which tend to appear after infection. Most people recover after 4 to 7 days without treatment, but a person with severe diarrhea may need hospital treatment.

Infection of *Salmonella typhimurium*, leads to the development of typhoid, or enteric fever. This disease is characterized by the sudden onset of a sustained and systemic fever, severe headache, nausea, and loss of appetite. Other symptoms include constipation or diarrhea, enlargement of the spleen, possible development of meningitis, and/or general malaise. Untreated typhoid fever cases result in mortality rates ranging from 12-30% while treated cases allow for 99% survival.

Materials and Methods

Sample Collection

Meat samples (Chicken meat) and Coleslaw ingredients/Vegetables (cabbage, Carrots, Green peas and Mayonnaise) were purchased from four different

markets in Owerri, Nigeria. These were: Relief Market, Orji Market, Ihiagwa Market and Obinze Market. Twenty (20) samples were collected from each market at random from different tables, amounting a total of 80 samples. Each sample was properly labeled in sterile polythene bags, put in an ice pack and taken to the Microbiology laboratory for sample preparation and analysis.

Sample Preparation

Samples were prepared on a bench previously swabbed with 70% ethanol and allowed to dry. The chicken samples were chopped into pieces and 20 g of each meat (chicken) sample was minced in a sterile blender with 180 ml of distilled water. The meat was placed in sterile containers for further analysis. Vegetables for the Coleslaw were prepared according to methods described by FCJ refugee center community kitchen (2008). Approximately 20 g of the Coleslaw sample was weighed out, dispensed into 180ml of water, placed in a sterile blender and blended. Each blended sample was placed in sterile Polythene bags for further analysis.

The leaf samples (Scent and *Uziza*) was sun-dried and placed in sterile blenders and blended into powdery form. 20g of each blended leaf samples was placed in sterile containers with 200 ml of distilled water.

Bacterial Identification

The identification was based on biochemical test based international software available at http://www.tgw1916.net/bacteria_logare_desktop.html. Selected biochemical tests was conducted and the results fed into the software and collected. The following set of tests are required for the identification of the test isolates.

Standardization of Isolates

The McFarland's (MF) Standard was prepared following the reaction between 1% Barium chloride and 1% Sulfuric acid standardized into MF standard 0.5, 1.0, 2.0, 3.0 and 4.0 (Vereecken *et al.*, 2000). The MF plot was obtained by plotting values obtained from the A_{500} readings against the predefined standard number of cells. The cells used in this work were harvested and washed; re-suspended and the absorbance was read at A_{500} . The Number of cells used was therefore calculated by interpolation.

Experimental Design

A General full factorial design (2^k) was carried out using three different food additives (Scent leaf, *Uziza* leaf and Salt) so as to ascertain the effect of each factor in singles and in synergy. The effect of four concentration levels of each spice was tested against the standardized inoculum. The spices concentrations include Salt (0%, 2%, 6% and 10%), Scent leaf (0 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$, 800 $\mu\text{g/ml}$ and 1200 $\mu\text{g/ml}$) and *Uziza* leaf (0 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$, 800 $\mu\text{g/ml}$ and 1200 $\mu\text{g/ml}$). All Percentage was expressed in weight percent.

Experimental set up for Microbial Inactivation

The growth of the test isolates on the meat sampled was studied at different temperatures so as to obtain specific temperatures that could inactivate the microorganisms over a selected and graded incubation period. In this study, a 24-hour inoculum of the test isolates was diluted using tenfold serial dilution and 1 ml of the 10^6 diluent was inoculated in minced chicken meat sample (10 g portions). The inoculated test isolates were mixed with the meat and the samples stored in polyethylene bags at different temperatures (-4°C , 4°C , 25°C , 35°C and 45°C).

Sampling and Cultivation

To determine the temperature dependent inactivation of the selected test isolates, samples of each storage temperature treatment at the time of inoculation, after 4 h, then 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h were taken for microbial analysis using the spread plate technique. Experiments that evaluated survival at each storage temperature was replicated. At each sampling interval, frozen samples were thawed at room temperature for 20mins and 10g quantities were subsequently diluted 1:10 in 0.9% peptone saline water. These were subsequently mixed for 20s and plated on selective agar media plates using the spread plate method. Incubation of the inoculated plates was done at the appropriate growth conditions for each organism.

Data Analysis for Model Prediction

Data was transferred to Microsoft® Excel 2016 for the primary analysis. GInaFiT software described by (Geeraerd *et al.*, 2005) was used to identify appropriate survival models that fit the dataset by least squares regression and a logarithmic form of the

Weibull Model was selected in order to build predictive models.

Statistical Analysis

Statistical analyses were performed using MINITAB 17 software. Several analysis of variance and covariance (ANOVA) was carried out to determine the differences that occurred around the mean of bacterial counts. The F-test was used to compare the goodness of fitting between the log-linear model and the Weibull Model at $P < 0.05$ (Geeraerd *et al.*, 2005). Mean and Standard Deviations were calculated for all data sets and the results were indicated as Mean \pm Standard Deviation. The Mean Sum of Square Error (MSE) in conjunction with The R^2 Values was used to determine the Goodness of Fit at $P < 0.05$.

Results and Discussion

The control of microorganisms, especially potential pathogenic species have raised immense concern in recent decades. The use of local food spices to control the growth of microorganisms have been attempted by several studies. This study was thus undertaken to model the effects of local food spices on the growth of *Salmonella typhimurium*.

This study was conducted using conventional microbiological methods. *Salmonella typhimurium* isolates were obtained from Meat and Coleslaw samples. To assess the population of *Salmonella* associated with the samples, plate count was done using pour plate method. The results for the enumeration of the *Salmonella* count shows that the Cfug to range from 5.0×10^3 to 1.18×10^4 . The *Salmonella* count of the Red meat samples ranged from 2.3×10^3 to 3.2×10^3 Cfug. The results indicates varying degree of contamination of the samples. To ascertain the statistical difference between the *Salmonella* load of the samples, an analysis of variance (ANOVA) was conducted. The ANOVA indicates the counts were significantly different from each other ($p < 0.05$). Similar results were obtained in a study by Morey and Singh (2012) aimed to investigate the presence of *Salmonella* in distributed chicken meat. In this study, 100 samples of chicken meat were selected and investigated for the presence of *Salmonella*. Each sample was cultured in selenite cystine medium and incubated at 37°C for 24 hours. Then the obtained colonies were cultured on MacConkey agar and *Salmonella-Shigella* agar.

Finally, biochemical and antibiogram tests were performed on isolated *Salmonella* samples. The results obtained showed that in total, 7 chicken samples (7%) were found to be contaminated with *Salmonella*. All of the isolated *Salmonella* samples were identified as *Salmonella enteritidis*. All of *Salmonella enteritidis* isolates (100%) showed the highest resistance to erythromycin and ampicillin antibiotics. All of the tested isolates (100%) showed sensitivity to gentamicin.

The Salad vegetables also had generally lower counts than the Chicken and Red meat samples with a range of 4.0×10^2 - 2.55×10^3 Cfug. Counts were also significantly different from each other by comparing their means using ANOVA at $P < 0.05$.

In a study undertaken by Ronoh(2011) aimed at assessing the prevalence of bacterial contaminants in meat, out of the 27 samples cultured from the slaughterhouse, 15 tested positive for pathogens. Of the 15, 4 (27%) were positive for *Staphylococcus aureus*, 6 (40%) for *Proteus vulgaris* and 5 (33%) for *Proteus rettgeri*. *Pseudomonas aeruginosa* was not isolated in all the samples of the slaughterhouse. From the 27 samples collected from butcheries, 24 were positive for pathogens. Of the 24, 7 (29%) were positive for *Bacillus subtilis*, 6 (25%) for *Proteus rettgeri*, 7 (29%) for *Proteus vulgaris* and 4 (17%) for *Pseudomonas aeruginosa*.

The *Salmonella* isolates were identified using series of biochemical tests and the "Advanced Bacteriology Identification Software" (ABIS). To model the effects of local food spices on the growth of *Salmonella typhimurium*, the bacterial isolates were standardized using McFarland's (MF) method. According to Vereecken (2000), McFarland Standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard. A McFarland Standard is a chemical solution of barium chloride and sulfuric acid; the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial suspension of known concentration.

McFarland's (MF) Standard was obtained following the reaction between Barium chloride and Sulfuric acid standardized into MF standard 0.5, 1.0, 2.0, 3 and 4.0. The MF plot was obtained by plotting values obtained from the A_{500} readings against the predefined standard number of cells. The equation below was used;

$$y = 6 \times 10^{-10}x + 0.0297$$

With R^2 Values of 0.9972. Where y = Optical density and x =cell concentration.

The OD readings obtained for the 24-hour cultures were standardized to McFarland's standard 0.8 which corresponds to 1.28×10^9 cells.

The results for the McFarland's (MF) Standard are presented in Table 1 and Figure 1. At McFarland's (MF) 0.5, $1.50E+08$ was obtained, $3.00E+08$ cells were obtained at 1 MF, $6.00E+08$ at 2 MF, $9.00E+08$ AT 3 MF, and $1.20E+09$ at 4 MF. The standardized inoculum was used to determine the single and combined effects of Scent leaf, *Uziza* and Salts on *Salmonella typhimurium*.

Table 1:McFarland's standard table for determination of McFarland's Standard plot

MF STd	Abs	Log N	No of Cells
0.5	0.108	8.176	1.50E+08
1	0.212	8.477	3.00E+08
2	0.352	8.778	6.00E+08
3	0.549	8.954	9.00E+08
4	0.700	9.079	1.20E+09

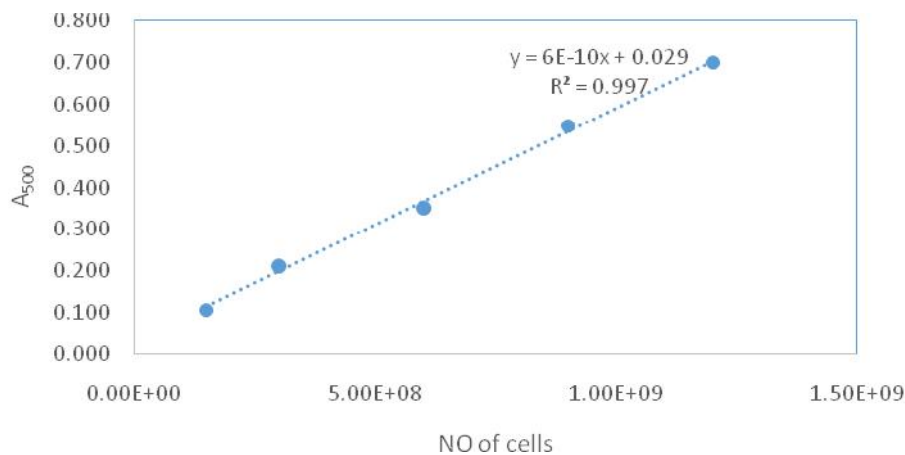


Fig 1:McFarland's standard plot for the determination of cell number of broth cultures used as standard inoculum

The main effect and combined effects of extracts of scent leaf, *Uziza*, and salts on *Salmonella typhimurium* was determined using a 2^k factorial plot. Figure 2 shows the main effect plots for the effects plot for the effect of the spices against *Salmonella typhimurium*. Results show that the *Uziza* extracts had mean stimulatory effect at 400 $\mu\text{g/ml}$ while the salt extracts had similar results at 4% salt concentration. However,

the mean inhibitory effect was obtained for scent leaf extracts at 400 $\mu\text{g/ml}$ and 800 $\mu\text{g/ml}$ but stimulatory at 1200 $\mu\text{g/ml}$. Also, at a concentration of 800 $\mu\text{g/ml}$, *Uziza* extracts had inhibitory effects while salt solution also had stimulatory effects at 8% concentration. At 12% salt concentration, the effect on mean growth was bactericidal demonstrated by a rapid decline below the initial bacterial population.

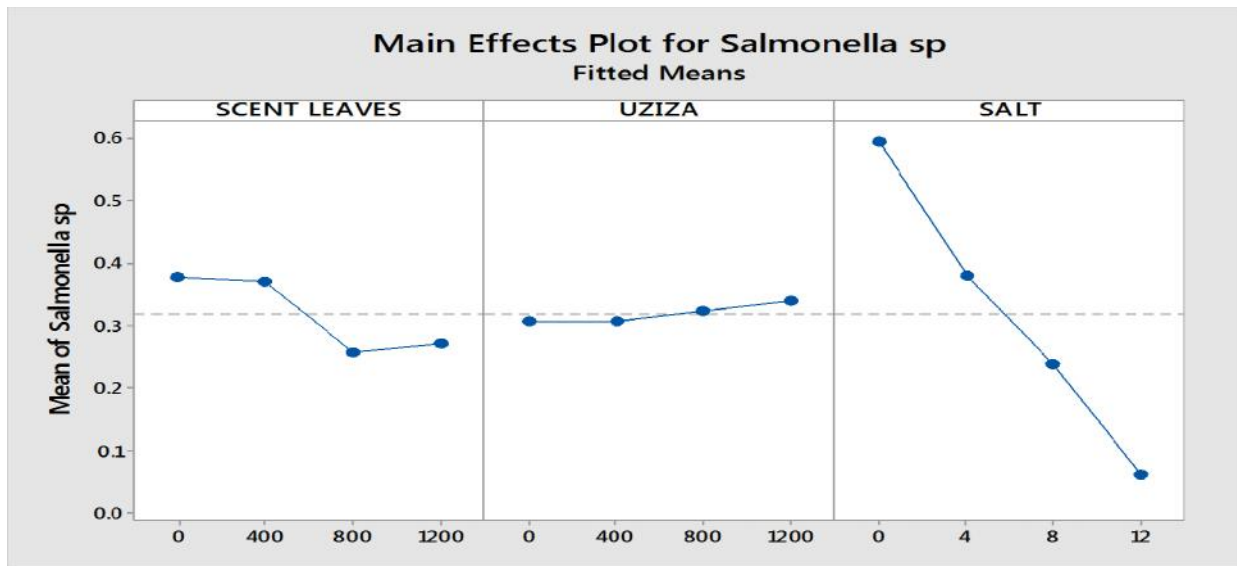


Fig 2: The activity of the selected food spices against *Salmonella typhimurium*

Figure 3 shows the interaction plot for the mean effects of extracts of the selected spices against *Salmonella typhimurium*. The interaction of salt x scent leaf and salt x Uziza had significant interactions causing inhibitory effects against *Salmonella typhimurium* as the concentration increases. The maximum interaction was observed at a concentration of 800µg/ml of Uziza and scent leaf and 8% salt

concentration resulting in the death of the isolate above 800µg/ml of Uziza and scent leaf and 8% salt concentration. Combinations of Uziza x salt and scent leaf x salt had weak interaction and effect resulted in a combined antagonistic effect using inhibition as a synergistic measure. Combinations of Uziza x scent leaf and scent leaf x Uziza had a mean stationary effect on the growth rate of *Salmonella typhimurium*.

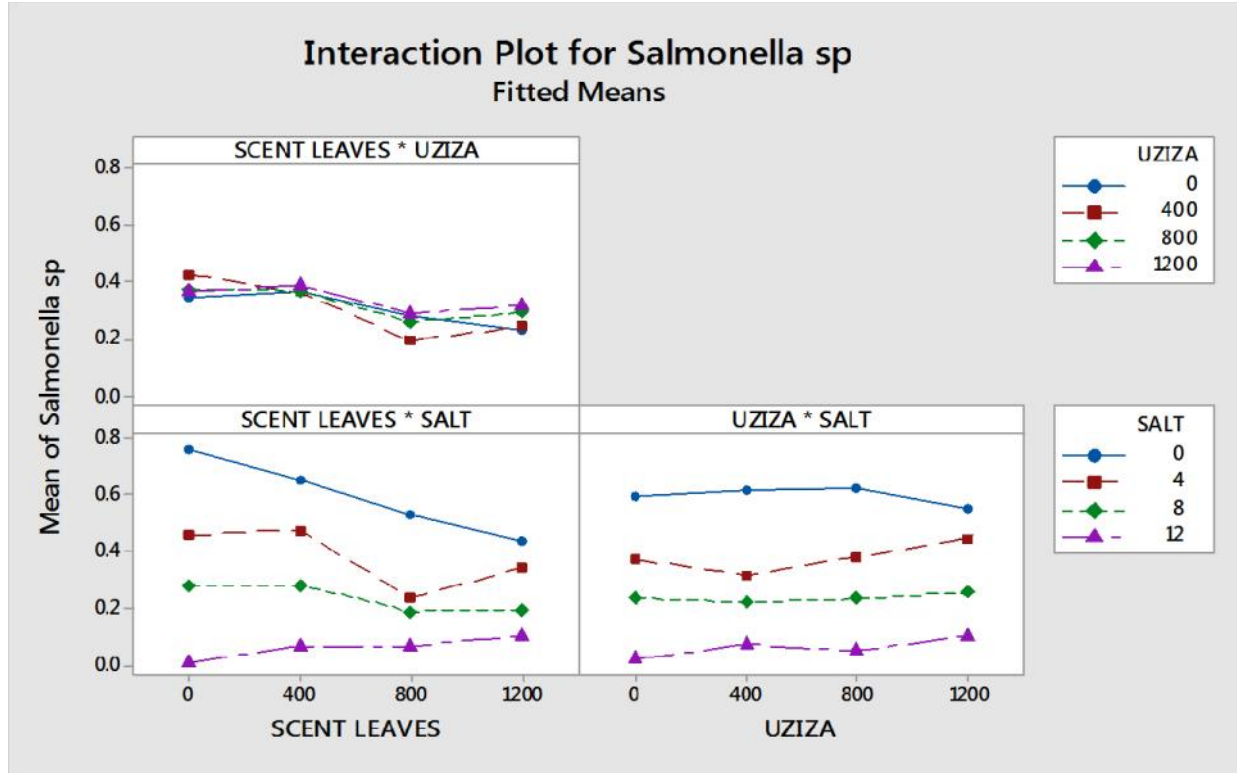


Fig 3: Combined effects of the selected food spices against *Salmonella typhimurium*

Model of prediction fitted into the Weibull model and Bigelow and Esty's model was used to evaluate the temperature dependent inactivation of *Salmonella*

typhimurium. At -4°C the data fitted into the Two Mixed Weibull distributions with an R² value of 0.9998. The fitted model is described in Fig 4.

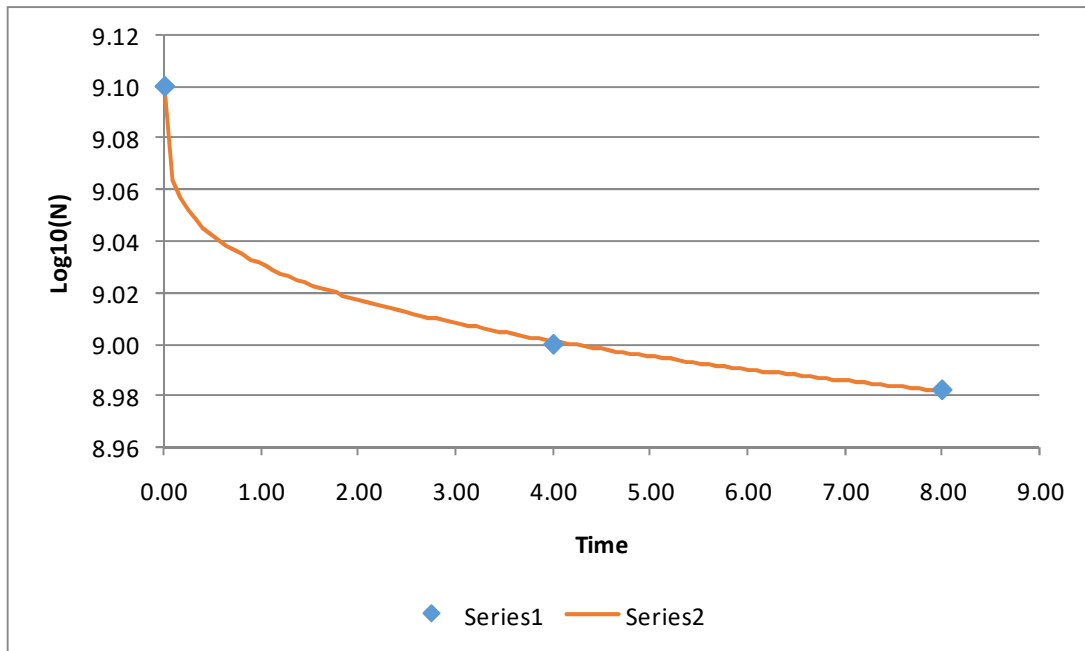


Fig 4: Two Mixed Weibull Distributions of Temperature-dependent inactivation of *Salmonella typhimurium* at -4°C

At 4°C and 25°C, the data is fitted into the Bigelow and Esty's Model also known as thermal death Model with an R² Value of 0.7153 and 0.9690 respectively. The fitted model is described in Fig 5 and Fig 6. This

model signifies a positive slopped model with k_{max} and LOG10 (N₀) values of -0.02 and 9.12; and -0.02 and 9.10; for 4°C and 25°C respectively.

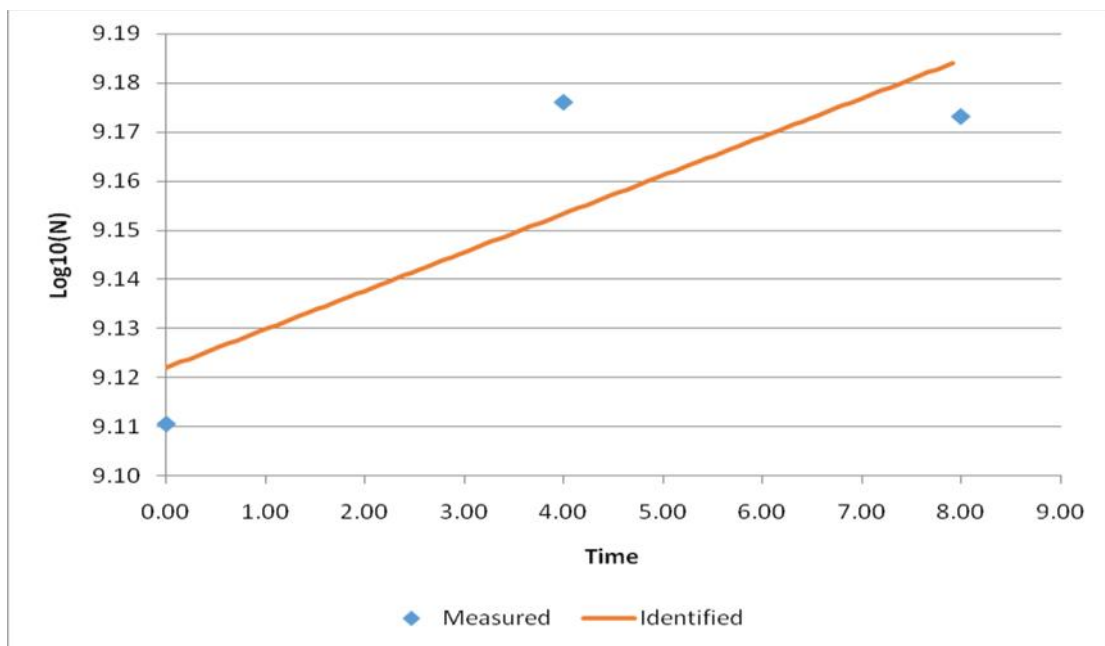


Fig 5: Bigelow and Esty's model fitting of growth of *Salmonella typhimurium* at 4°C

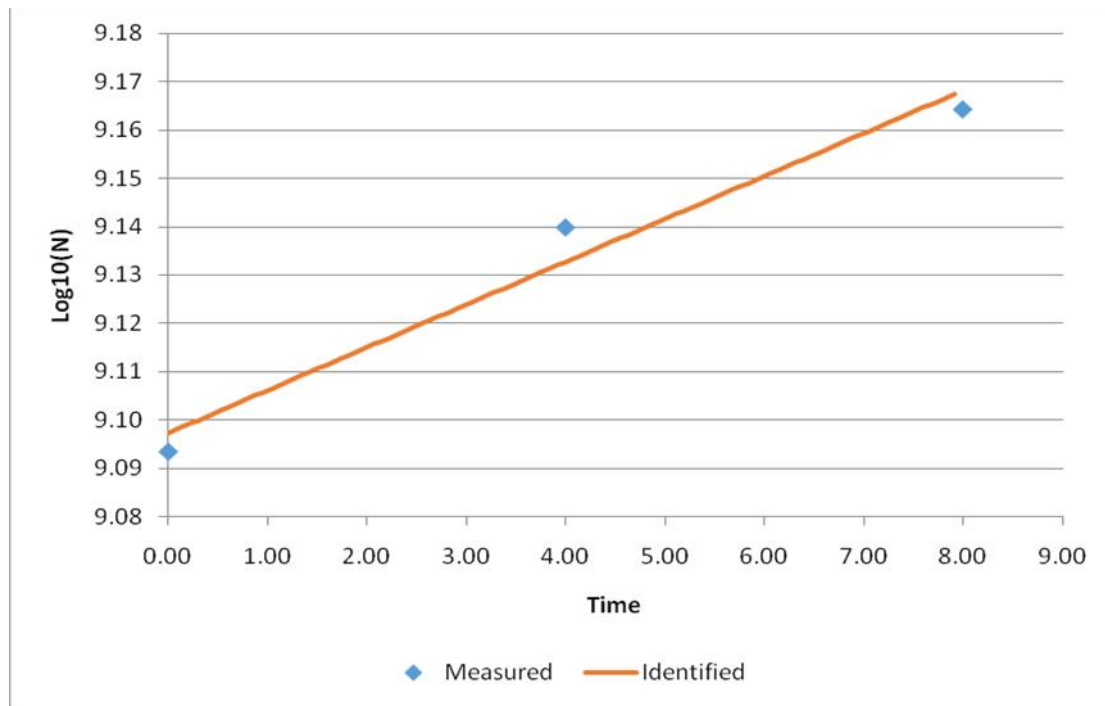


Fig 6: Bigelow and Esty's model fitting of growth of *Salmonella typhimurium* at 25°C

This signifies that the inactivation of the isolate is not possible at near room temperature and indicates the risk associated with samples displayed openly at market temperatures. Considering the modeling of the growth of the microorganisms at -4°C and 4°C, it is important to note that values of alpha, delta and β are only derivable for inactivation Models with a negative slope as reported by Morey and Singh (2012). Temperature and time of exposure were shown to play pivotal role in the cidal effects of the extracts on the test isolates.

Conclusion and Recommendations

The reported inactivation models depicts that it is possible to set standardized critical control points in the production line if *Salmonella typhimurium* is suspected as a contaminant. In addition, this work has demonstrated that the tested spices have single and combined effects against tested isolates. However, the progression for their single effects follows the trend from salt, scent leaves to *Uziza* leaves. However, there is a need to extract the active ingredients/phytochemicals in the spices and test them in their purified form.

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	Website: www.ijarbs.com
	Subject: Food Microbiology
Quick Response Code	
DOI: 10.22192/ijarbs.2021.08.11.006	

How to cite this article:

Oyadougha T.W., Chinakwe E.C. and Braide W. (2021). Modelling the effects of local food spices on the growth of *Salmonella typhimurium*. *Int. J. Adv. Res. Biol. Sci.* 8(11): 46-54.
DOI: <http://dx.doi.org/10.22192/ijarbs.2021.08.11.006>