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ORIGINAL RESEARCH ARTICLE

Preservative Properties of Antimicrobial Peptides of Germinated Seeds of *Pisum sativum* (Garden Pea) On *Roselle* Flowers (*Hibiscus sabdarifa* L.) Drink

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ABSTRACT
The demand for natural and effective food preservatives has increased due to the growing concern over the safety and quality of processed beverages. Antimicrobial peptides have gained significant attention as natural alternatives to synthetic preservatives, offering potential benefits such as broad-spectrum activity and lower toxicity. Germinating seeds of *Pisum sativum* were harvested on days 2, 4, 6 and 8 and peptides were extracted and purified using Ammonium Sulphate Precipitation at 70% saturation, Dialysis, Gel filtration and the peaks were obtained. The molecular weight of peptides was determined using SDS-PAGE while Six (6) Bacteria and Four (4) Fungal species were isolated and characterized using standard procedures. The highest protein concentration was obtained with germinated peas day four (P_4) with a value of 1.7866mg/mL, while the highest zone diameter of inhibition was recorded with peptide of germinated pea seeds day six fraction three ($P_{6:3}$) with a value of 17.000±0.577mm against *B. cereus*. The least MIC value, expressed in protein concentration (mg/mL), was obtained as 0.0253mg/mL with 100% concentration of the peptide of germinated peas on day four (P_4). All four (4) fungal species recorded no activity/zone diameter of inhibition. Sanitizing activity of the peptides on the '*Zobo*' drink adjusted to pH 2.3, at 0hr, 24hr, 48hr and 72hrs was carried out, and all the purified peptides extended the shelf life of the drink except for peptide of germinated peas on day two. Sensory evaluation equally indicated that the drink was preserved with 10% ($P_{4:4}$) at pH 2.3, being the most preferred treatment by the judges with a percentage likeness of 72.22% after 72hrs of storage. Findings from this study highlight the potential of these peptides in developing innovative and safe preservation techniques for extending the shelf life of local African beverages. This study further offers promising insights into the application of *Pisum sativum* peptides in preserving traditional drinks like *'Zobo.*'

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INTRODUCTION
The increasing human population and the demand for Healthy and nutritious food imposes food security challenges worldwide (Bajpai *et al.*, 2012). It has been reported that an estimated 600 million, which means almost 1in10 people in the world, become ill after consuming contaminated food that can cause notably severe diarrhoea, and 420,000 die every year (WHO, 2017). To face this threat, it is imperative to ensure food safety throughout the entire food chain, from the production to the consumption of the end-products (WHO, 2014).

Microbial resistance is one of the main fears of modern medicine, and it has driven the search for newer, potent antimicrobial agents to replace conventional antibiotics (Hassan *et al.*,2012). Hence, both pharmaceutical and food industries are looking for safer, new types of antimicrobials from natural sources (Boisvert *et al.*, 2015). The search for an alternative to chemical preservations has led to defined novel methods like bio-preservation that ensure food safety (Pisoschi *et al.*, 2018). Antimicrobial peptides (AMPs) such as Bacteriocins have attracted particular attention as their producer organisms could obtain GRAS (generally recognized as safe) status and are naturally present in many food products (Tahiri *et al.*, 2009). Their low toxicity, thermostability or high specificity make these molecules of clear interest to the food industries (de Castro and Sato, 2015). While plant-derived peptides are well-documented for their antimicrobial properties, there is a noticeable gap in research regarding their application in the preservation of traditional African beverages, including the 'Zobo' drink. Also, their specific use in enhancing the shelf life and safety of traditional beverages is largely underexplored,

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especially in the context of the 'Zobo' drink—a widely consumed beverage made from hibiscus petals. This represents a novel research frontier and justifies the aim of the present study to isolate, purify and characterize the plant antimicrobial peptides sourced from germinating seeds of *Pisum sativum* and to test its efficacy in Biopreservation of *zobo* drink.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

All samples were first identified in the field using standard keys and descriptions using the latest phylogenetic classification (Chase *et al.*, 2016). Further confirmation and authentication of Peas (*Pisum sativum*) BUKAN 0571 and Roselle (*Hibiscus sabdariffa L.*) BUKAN 0040 was carried out at the Herbarium of the Department of Plant Biology, Bayero University Kano, Nigeria.

Seed Germination

The optimal germination process was performed according to the Rules of the International Seed Testing Association (Anon, 1999), where 800 visibly healthy seeds of Peas (*Pisum sativum*) were used for the germination assay. Seeds were first surface sterilized and soaked in distilled water for 12 hours and later distributed in 10 trays. The seeds were spread on a moist sheet of filter paper covered with another sheet of moist filter paper and put into a germination chamber under environmentally controlled conditions: 20^0C, 8 hours of light per day exposure. Germinating seeds were collected on days 2, 4, 6 and 8. The germinated seeds were further freeze-dried, milled and passed through a sieve of 0.5 mm. The germinated pea seed flour was stored at 4^oC until analyzed.

Extraction of Proteins for Antimicrobial Peptides

The 10g of dried milled seeds were treated with 100 ml (1:10) of Phosphate buffer pH 7.5, 0.1M solution at 4^0C. The mixture was frozen and thawed three (3) times and centrifuged at 10000 rpm for 10 mins and stored at 4^oC in a freezer (Pasha *et al.*, 2016).

Estimation of Total Protein by Lowry's Method

Different dilutions of Bovine Serum Albumin (BSA) solutions were prepared, and 0.2 ml peptide solution was taken out using a pipette to different test tubes. Two (2) ml of alkaline copper sulphate reagent (analytical reagent) was added and incubated at room temperature for 10 mins. 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 min, and the colourimeter was adjusted to zero. The optical density was taken (measure the absorbance) at 660 nm, and the graph of absorbance against protein concentration was plotted to get a standard calibration curve (Chang and Zhang, 2017)

Purification and Characterization of Antimicrobial Peptides using Ammonium Sulfate Precipitation Method

Antimicrobial peptides can be concentrated through a process called salting-out using ammonium sulphate as a common reagent. The supernatant of the Phosphate Buffered Saline processed samples containing soluble portions of the milled seeds were treated with 70% ammonium sulfate maintained at 4^oC for 20 hours. The precipitate was recovered by centrifugation, and the supernatant was further refrigerated and centrifuged at 10000 rpm for 20 minutes. Supernatants were removed by re-spinning briefly to clear the remaining ammonium sulfate (Anna and Sanaa, 2014).

Dialysis

The precipitated samples were dialyzed to remove unwanted molecules from the samples using a membrane filter with 2-50,000kDa pore size (Valery *et al.*, 2014).

Gel filtration Chromatography

The dissolved ammonium sulfate precipitate was further purified by gel filtration chromatography using SephadexG-75 with sodium acetate buffer (pH 5). The eluted samples were quantified for the presence of peptides spectrophotometrically at an absorbance of 280 nm. The protein concentrations were plotted against the number of eluted fractions and peak concentrations, determined (Rehman and Khanum, 2011).

SDS-PAGE

The SDSPAGE Using ExpressPlusTM PAGE Gels was carried out according to the manufacturer's specifications. This was done to determine the molecular weight of the isolated peptides (Rehman and Khanum, 2011).

Isolation and Identification of Bacterial and Fungal species from spoiled foods

A total of six (6) bacteria and four (4) fungal species were isolated from stale white rice, 'zobo' drink, 'kunun zaki' and 'jollof' rice. The organisms from different culture media were examined physically for their characteristic appearance and microscopy. Biochemical tests for the Bacterial species were carried out, while Lactophenol Cotton Mount was also done for the fungal isolates. Isolates were subjected to molecular analysis and confirmed after sequencing of their genetic material (Mahuku, 2004).

Susceptibility Test

The antimicrobial activity was determined using agar well diffusion. Inoculum was prepared by adding 0.1 ml of overnight cultures of each organism into 20 ml of sterile Lactose broth medium and incubated for 8 h to

standardize the culture of 10^8cfu/ml. Wells of 4 mm diameter were bored on Mueller-Hinton agar plates to which 100µl of culture was spread, and extracted peptides with 100% concentrations of the peptides as obtained from the gel chromatography were inoculated into the wells. The plates were incubated for 24 hours at 37^0C. The zone diameter of inhibition was measured, and for each combination of peptide and bacterial/fungal species, the experiment was performed in triplicate, and the sample size was determined based on a power analysis to ensure sufficient sensitivity in detecting differences between treatments (Murugan *et al.*, 2007).

Minimum Inhibitory Concentration in Culture Media

Minimum inhibitory concentration (MIC) values of peptide isolated from seeds of *Pisum sativum* were determined by broth dilution assay. The fresh culture of spoiled food isolates were inoculated into LB broth and 1ml of antimicrobial protein preparation12.5% (0.125mL protein +0.875mL normal saline), (25% (0.25mL protein+ 0.75mL normal saline), 50% (0.5mL protein 0.5mL normal saline), and 100% (no addition of normal saline) was added and incubated at 37^oC for 24 hrs. Results were expressed in protein concentration (mg/mL), and for each combination of peptide and bacterial/fungal species, the experiment was performed in triplicate (Murugan *et al.*, (2007).

Determination of Physical, Chemical and Biochemical Properties of the Peptides

pH and Heat Treatment

The method described by Motta and Brandell (2008) was adopted. While keeping the temperature constant, the different concentrations of the peptides were dispensed into test tubes and their pH was checked. The peptides were then adjusted to various pH values (3-11) by adding drops of NaOH and HCL while checking the pH until the desired acidity and alkalinity were achieved. The solutions were then incubated at 30^0C for 3 hours then residual activity was checked using Agar well diffusion. Heat stability was also examined by treating the peptide preparation at a temperature of 30^0C, 40^0C, 50^0C, 60^0C, 70^0C, 80^0C, 90^0C, 100^0C and 121^0C for 15 minutes, according to the method described by Motta and Bendell (2008). The different concentration of the peptide solutions was dispensed into test tubes and placed in an oven, while adjusting the temperature to the desired value for 15mins. After cooling, the peptide antimicrobial activity of the different heat treatments was then evaluated using agar well diffusion. Again, the experiment was conducted in triplicates.

Effect of Enzyme and Surfactants

The sensitivity of the peptides to enzymes was tested (in triplicate) on the cell-free supernatants (pH 7) of 24hrs culture incubated at 30^0C and treated for 2 hours with 0.1mg/mL and 1.0mg/mL final concentration of the enzyme trypsin (Hi Media Laboratory Pvt Ltd. India) (Rajarum *et al.*, 2010).

Preservative Studies

Preparation of roselle calyx drink ('zobo')

The production of 'zobo drink' was carried out according to methods described by Aramide *et al.* (2009) and modified by Bukar (2012). Dried calyxes of *Hibiscus sabdariffa* were picked and washed, and ten (10) grams were weighed and boiled with one litre of clean water for 10 minutes and allowed to cool. The juice was extracted by squeezing the boiled calyxes and then sieving the thick red suspension using a clean, sterile sieve. Sixty grams (60g) of sugar was added, stirred and cooled. No flavour or preservative was added. The experiment was performed in triplicate, and the sample size was determined based on a power analysis to ensure sufficient sensitivity in detecting differences between treatments.

'Zobo drink' Treatments

Twenty millilitres (20mL) of "zobo drink" were filled in each of a set of sterile bottles of 30mL capacity. Five different treatments were carried out as follows (Ali and Bukar, 2018)

A. 20ml of "zobo" drink with 2ml (10%) (v/v) Peptide

B. 20ml of "zobo" drink" + 1ml (5.0%) (v/v) Peptide

C. 20ml of "zobo" drink" + 0.5ml (2.5%) (v/v) peptide

D. 20ml of "zobo" drink" + 0.25ml (1.25%) (v/v) peptide

E. 20ml of "zobo" drinks" + 0.1mg (0.5%) (w/v) sodium benzoate.

F. 20ml of "zobo" drink" left untreated

Enumeration of Aerobic Mesophilic Bacterial Count for Evaluating Sanitizing Activity of Peptide on 'Zobo' drink.

Bacterial Count

Serial dilution of the various treatments of the 'zobo' drink was carried out up to 10^{-5} at 0hr, 24hrs, 48hrs and 72hrs. From the serially diluted samples, 1ml from 10^{-3} dilutions were transferred into duplicated petri dishes each. This was followed by Pouring 15ml of molten Nutrient agar. The plates were swirled and allowed to solidify and incubated at 37^oC for 24 hours. The colonies formed were

counted and multiplied with the dilution factor (FAO, 2010).

Determination of pH, Temperature

The pH of the various samples of zobo drink was determined using a pH meter (Jenway). Before use, the pH meter was calibrated using a pH 7.0 solution. A standard thermometer was also used to determine the temperature.

Determination of Titratable Acidity

The titrimetric determination of acidity (expressed as lactic acid %) of the different zobo drink samples was accomplished according to AOAC (1990). Ten-millilitre sample was pipetted into a 100 mL conical flask. The pipette was washed with 10 mL water. Two drops of phenolphthalein were then added. The sample was then titrated with 0.1 N NaOH until a stable blue colour had formed. The titration figure was divided by 10 to obtain the percentage of lactic acid.

Shelf-Life Extension of Roselle calyx 'Zobo' Drink

The experimental set-up for sanitation of roselle calyx 'zobo drink' was replicated. However, in this experiment, the number of days for each of the treatment A – F to deteriorate were studied and recorded (Peryam, 1998).

Sensory Evaluation of Roselle calyx 'Zobo' Drink

Organoleptic parameters such as taste, colour, texture, and odour were assessed by a panel of ten (10) judges. Scores were graded on a hedonic scale (Peryam, 1998).

Statistical Analysis

Data generated from the sanitizing activity tests and the scores generated based on the assessment of judges (from the preservative experiments) using the Hedonic scale were statistically analyzed using Analysis of Variance (ANOVA) at 5% probability level using the software package developed by Microsoft Corporation.

RESULTS

Percentage Germination of Seeds

Figure 1 shows the result for the germination of Garden peas (*Pisum sativum*) seeds designated as P. The number of seeds germinated increased as the number of days increased so also the percentage germination. For Beans (P), the eighth (8^{th}) day recorded the highest number of germinated seeds, with a total of seven hundred and fourteen (728) seeds out of the initial 800 seeds. This is equivalent to percentage germination 91 .25%.

Crude Sample

Table 1 shows the concentration of the protein for Germinated Beans at days 2,4,6, and 8, designated as P_2, P_4, P_6, and P_8, which were determined after checking the absorbance of the unknown sample at 660nm. Using the straight-line graph (y=mx+c) where c is the intercept, which was 0, m is the slope, which was 0.454, y is the average absorbance at 660nm, and x is the concentration which was calculated. The highest concentration was obtained with a crude sample of germinated Peas on day 8, while the lowest protein concentration was obtained with germinated peas on day 2.

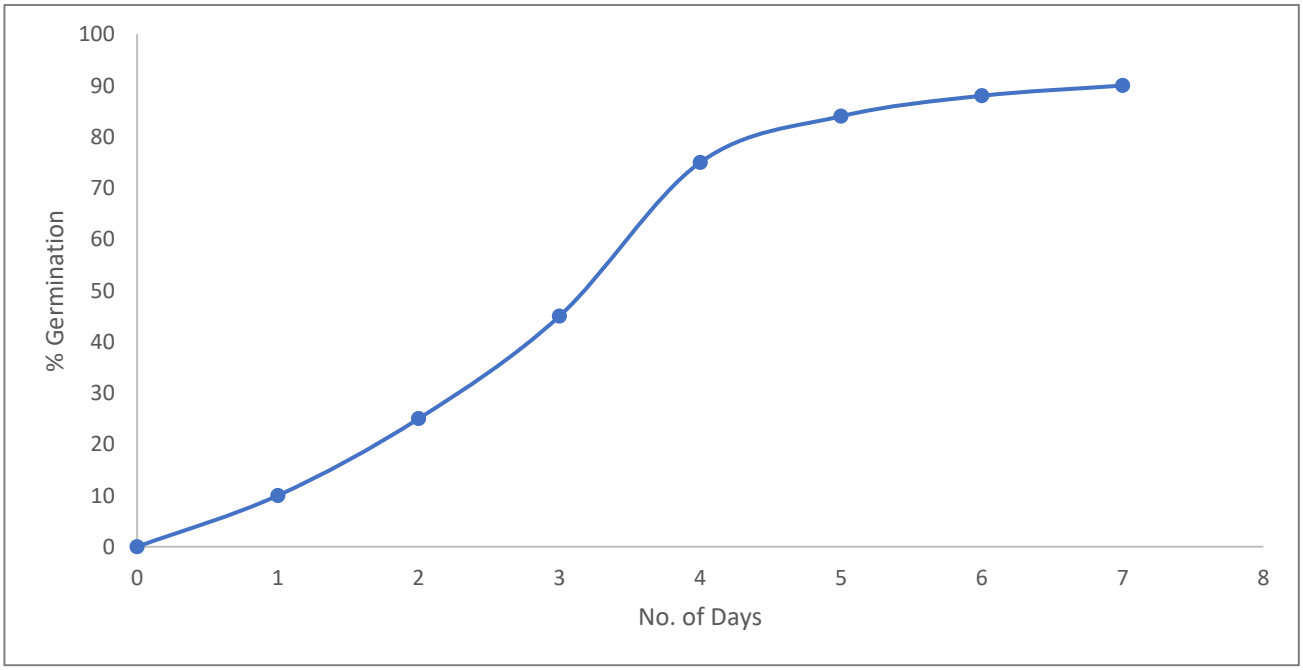

Figure 1: Percentage of Pea Seeds germinated in Eight (8) days

Table 1: Protein Concentration of Crude Sample

Beans	Average Absorbance	Protein conc(mg/ml)
P_2	0.912	2.009
P_4	1.019	2.244
P_6	0.987	2.174
P_8	0.989	2.178

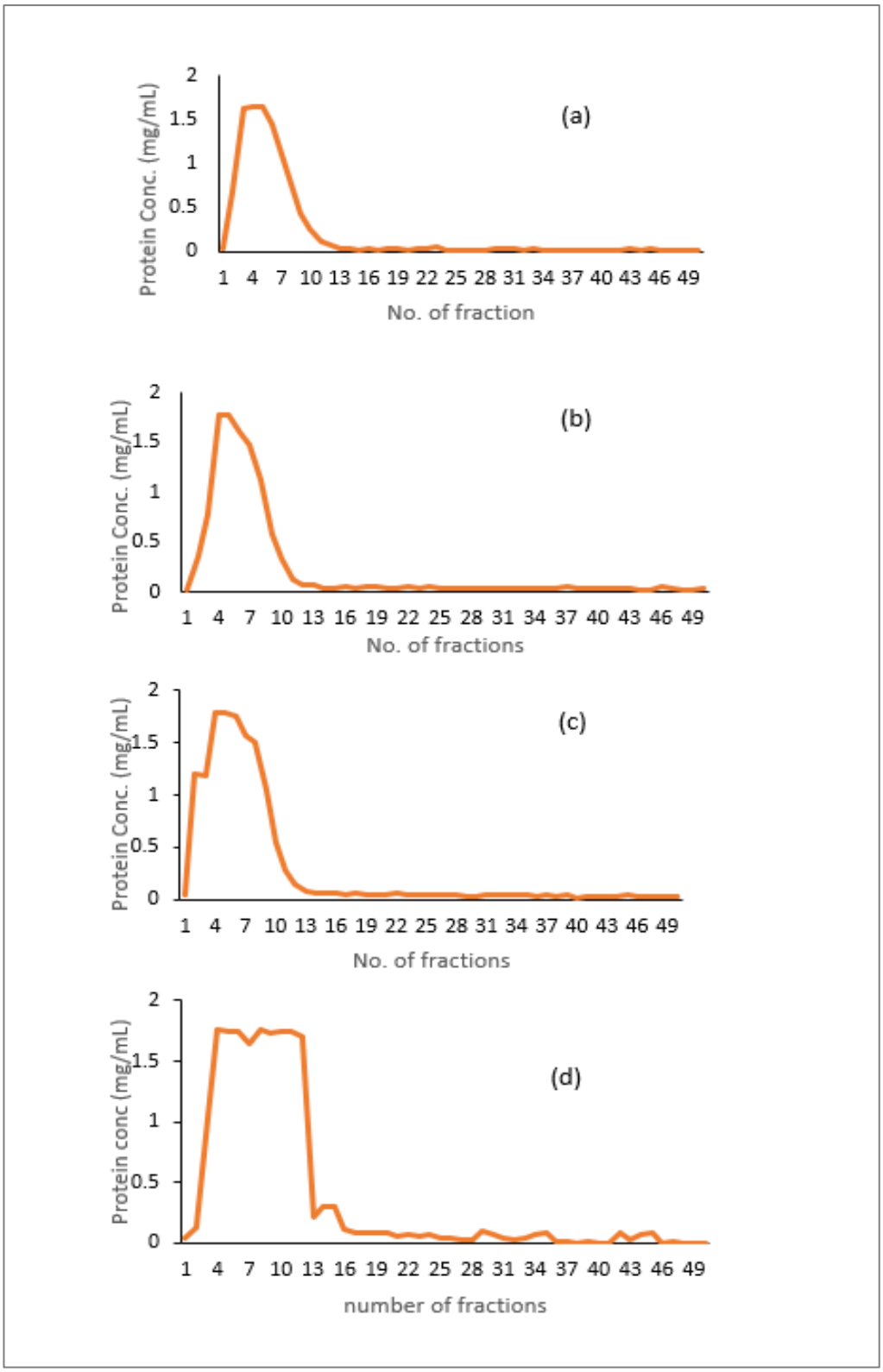

Figure 2: A spectrophotometric analysis showing the number of Fractions (50), Protein concentration (mg/mL) and Peak concentrations of the Purified Peptides of Germinated peas where a = day two (P_2), b = day four (P_4), c = day two (P_6) and d = day two (P_8)

Gel filtration Chromatography

The ammonium sulfate precipitate was further purified using gel filtration chromatography, where fifty (50) fractions were obtained for each of the four (4) partially purified samples (Figure 2). The peptide concentration of the eluted fractions was measured spectrophotometrically at 280nm. The highest peptide concentration was recorded for germinated pea day 6 (P_6) with a concentration of 1.7866mg/mL, while the lowest was recorded for germinated pea day 8 (P_8) with a concentration of 0.0016mg/mL. The graph of absorbance against the number of fractions was plotted, and all peak fractions were further subjected to antimicrobial assay.

Molecular Weight Determination Using SDS-PAGE

The peptide from germinated pea seed on day two fraction four ($P_{2:4}$) was found to be approximately 10KDa, while that of germinated pea seed on day four fraction four ($P_{4:4}$) was found to be approximately 12KDa (Figure 3). The proteins from germinated pea seed on day six fraction two ($P_{6:2}$) and day eight fraction three ($P_{8:3}$) were both found to be approximately 7.5KDa. The highest molecular weight obtained was found to be 12KDa obtained from proteins of germinated pea seeds of day four fraction four ($P_{4:4}$).

Isolation and Identification of Bacterial Foodborne Pathogenic/Spoilage Bacteria

Isolation and identification of bacterial foodborne pathogens/spoilage organisms was carried out using the Cultural, Biochemical and Molecular techniques (Table 2). Results revealed five (5) different bacterial and three (3) different fungal species. They include *Salmonella, S. aureus, Listeria, bacillus cereus, E. coli* and *Pseudomonas,* while the fungi include *A. niger, A. fumigatus, S. cerevisiae and Candida albicans*

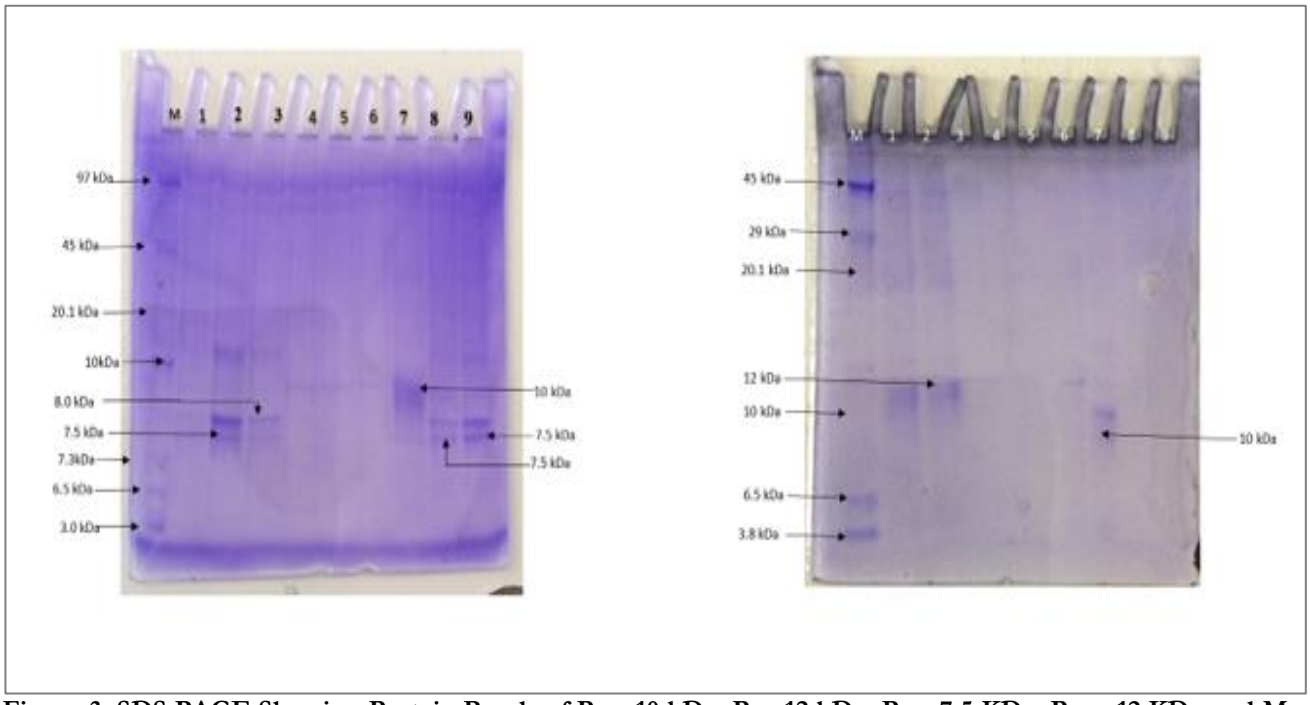

Figure 3: SDS PAGE Showing Protein Bands of $P_{2:4}$=10 kDa, $P_{4:4}$=12 kDa, $P_{6:2}$=7.5 KDa, $P_{8:3}$= 12 KDa and M= protein ladder.

Table 2: Summary of 16S rRNA/18S rRNA Sequencing results of the Isolates to identify the Closest Homologs

Sample ID	Sequence bp	Identity of the closest homologs of the sequence	% identity	Accession Nº of the closest homolog
E	727	*Escherichia coli* strain N4	93.01 %	MF754146.1
B1	752	*Bacillus cereus* OOA	99.73 %	OR702892.1
S	754	*Salmonella* enterica subsp. Enteric serovar *typhi*	100 %	OR078809.1, AB855737.1
Sa	763	*Staphylococcus aureus* B3	98.69%	MT154229.1
P2	997	*Pseudomonas aeruginosa*	96.66%	MH470331.1
5-H	545	*Aspergillus niger*	98.82%	MT620753.1
7-H	541	*Aspergillus fumigatus*	97.97%	MT645322.1
J	471	*Saccharomyces cerevisiae* isolate HAM3	99.79%	OQ318157.1

Antimicrobial activity of purified peptide of germinated Peas seeds

The antimicrobial activity of the purified peptides of germinated peas against the foodborne /spoilage bacteria and fungi revealed varied zone diameters of inhibition (Table 4). The highest zone diameter of inhibition of 17.0000±1.154mm was obtained against *B. cereus*, while the highest zone diameter of inhibition for peptides of germinated peas day four (4) was found to be 16.667±0.8819mm against *E. coli* and *Salmonella Typhi*, 17.000±0.5773mm was obtained with 1.1926mg/mL concentration of fraction three (3) against *B. cereus* and 17.000±0.5773mm was obtained with 1.0173mg/mL concentration of fraction three (3) against *B. cereus* With fungi, no activity/zone diameter of inhibition was recorded against all the fractions.

Minimum Inhibitory Concentration (MIC) of Purified Peptides of Germinated Peas Seeds

The minimum inhibitory concentration (MIC) of the peptide of germinated peas day 2 (P_2) against test organisms, expressed in protein concentration (mg/mL), was found to be 0.0253mg/mL against *Salmonella Typhi*. Similarly, the MIC of the peptides of germinated pea seeds day 4 (P_4) against test organisms shows that MIC was found to be 0.0200mg/mL, while the MIC of the peptides of germinated pea seeds day 6 (P_6) against test organisms was found to be 0.050mg/mL. Lastly, the MIC of the peptides of germinated pea seed day 8 (P_8) against the test organisms was found to be 0.0360mg/mL.

Sanitization Activity of "zobo drink."

Sanitization activity (Figure 4) of 'zobo' drink at pH 2.3, treated with the peptide of germinated peas seed day 2 fraction 4, ($P_{2:4}$), germinated peas seed day 4 fraction 4, ($P_{4:4}$), germinated peas seed day 6 fraction 2, ($P_{6:2}$) and germinated peas seed day 8 fraction 3, ($P_{8:3}$) was carried out at 0, 24, 48 and 72hrs. The results are expressed in *log* increase and or *log* reduction.

Biopreservative Activity of Peptides on 'zobo' drink

The sensory evaluation (Table 9 and 10), expressed as percentage likeness, using a nine-point hedonic scale, of the 'zobo' drink treated with peptides of germinated peas seeds at pH of 2.3 was carried out at 0hrs, 24hrs, 48hrs, and 72hrs for germinated peas seed day 2 fraction 4, ($P_{2:4}$), germinated peas seed day 4 fraction 4, ($P_{4:4}$), germinated peas seed day 6 fraction 2, ($P_{6:2}$) and germinated peas seed day 8 fraction 3, ($P_{8:3}$).

Table 3: Antimicrobial Activity of Crude Extracts of Germinated Bean Seeds

Organisms	P_2	P_4	P_6	P_8
S. aureus	4.000	12.000	8.000	12.000
SD	±0.0000	±0.3333	±0.0000	±0.3333
S. typhi	10.000	4.000	10.000	8.000
SD	±0.0000	±0.0000	±0.0000	±0.0000
E. coli	8.000	11.000	10.000	8.000
SD	±0.0000	±0.6667	±0.0000	±0.0000
Listeria spp	10.000	8.000	12.000	10.000
SD	±0.0000	±0.0000	±0.3333	±0.0000
A. fumigatus	4.000	4.000	4.000	4.000
SD	±0.0000	±0.0000	±0.0000	±0.0000

P_2= Germinated Peas Day 2, P4= Germinated Beans Day 4, P6= Germinated Beans Day 6, P8= Germinated Beans Day 8. Size of well = 4mm. SD= Standard Deviation

Table 4: Antimicrobial activity of purified peptide of germinated Peas

Organisms	1 0.050mg/Ml	2 1.204mg/mL	3 1.192mg/mL	4 1.786mg/mL	5 1.783mg/mL	6 1.759mg/mL	7 1.569mg/mL	8 1.500mg/mL	9 1.048mg/mL
S. typhi	8.6667 ±0.6667	13.0000[a] ±0.5773	12.0000 [ab] ±0.5773	13.6667 [a] ±0.6667	17.0000[a] ±0.5773	16.3333[a] ±0.8819	11.3333[a] ±0.8819	11.3333 [a] ±0.6667	10.3333 [a] ±0.3333
S. aureus	9.0000[a] ±0.0000	14.0000[a] ±0.5773	12.3333 [ab] ±0.3333	12.6667[ab] ±0.6667	11.0000 ±0.5773	11.0000[ab] ±0.5773	10.3333 [a] ±0.3333	10.3333 [a] ±0.3333	10.0000 [a] ±1.1547
Listeria spp	9.0000[a] ±0.0000	10.6667[a] ±0.6667	10.3333 ±0.6667	12.6667[ab] ±0.6667	14.3333[ab] ±0.3333	11.6667[ab] ±0.6667	11.0000[a] ±0.5773	10.0000 [a] ±0.5773	10.0000 [a] ±0.5773
E. coli	10.6667[a] ±0.6667	10.0000 ±0.0000	13.3333 [ab] ±0.6667	13.3333 [a] ±0.6667	16.6667[a] ±0.6667	12.3333[ab] ±0.3333	10.666 [a] ±0.3333	10.6667 [a] ±0.3333	10.6667 [a] ±0.6667
P aeruginosa	9.6667[a] ±0.6667	12.3333[a] ±0.8819	13.6667[ab] ±0.6667	13.0000[a] ±0.5773	11.3333 ±1.2018	11.0000 [ab] ±0.0000	4.0000 ±0.0000	10.0000 [a] ±0.0000	10.0000 [a] ±0.0000
B. cereus	9.3333[a] ±0.6667	14.0000[a] ±0.5773	17.0000[a] ±0.5773	16.6667[a] ±0.6667	13.0000 [ab] ±0.5773	12.3333[ab] ±0.3333	9.3333 ±0.6667	4.0000 ±0.0000	4.0000 ±0.0000
A. fumigatus	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000
A. niger	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000
Candida spp	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000
S cerevisea	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000
Control (50% Sodium Benzoate)	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000	4.0000 ±0.0000

Key: Size of well = 4mm. Control= Sodium Benzoate at 1% concentration. SD = Standard Deviation, Values are mean ± SD

Table 5: Minimum Inhibitory Concentration (MIC) in mg/ml of purified peptide of germinated Peas

	Organisms	S. typhi	S. aureus	Listeria spp	E. coli	P. aeruginosa	Bacillus cereus
1	0.0253mg/mL	0.0253	0.0253	0.0253	0.0253	0.0253	0.0253
2	0.674mg/mL	0.337	0.337	0.168	0.674	0.337	0.674
3	1.622mg/mL	0.4055	0.4055	0.4055	0.811	0.811	-
4	1.653mg/mL	0.4132	0.8265	0.8265	0.8265	0.8265	0.8265
5	1.649mg/mL	0.8245	0.4122	0.8245	0.8245	0.4122	1.649
6	1.455mg/Ml	0.363	0.727	-	0.727	1.455	1.455
7	1.102mg/mL	0.551	0.551	0.551	0.275	1.102	1.102
8	0.777mg/mL	0.777	0.388	0.388	0.388	-	0.777
9	0.428mg/mL	0.428	0.428	0.428	0.214	-	0.428

Table 6: Effect of pH on the purified Peptides of Germinated Peas seeds

Org	*S. typhi*	*S. aureus*	*Listeria* spp	*E. coli*	*P. aeruginosa*	*B. cereus*
pH3	9.6667±0.3333	7.3333±0.3333	6.0000±0.0000	12.0000±1.0000	11.6667±0.3333	10.6667±0.6667
4	14.0000±0.0000	10.6667±0.6667	10.6667±0.6667	12.6667±0.3333	11.3333±0.6667	12.0000±0.0000
5	11.3333±0.6667	10.6667±0.8819	9.6667±0.3333	10.0000±0.0000	10.6667±0.6667	10.3333±0.3333
6	10.6667±0.6667	9.6667±0.3333	10.0000±1.0000	9.3333±0.3333	9.6667±0.3333	9.3333±0.6667
7	14.6667±0.6667	12.3333±0.3333	13.3333±0.3333	11.3333±0.3333	10.6667±0.6667	11.3333±0.3333
8	11.6667±0.3333	7.3333±0.3333	9.3333±0.3333	10.3333±0.6667	9.0000±0.0000	9.3333±0.3333
9	9.3333±0.6667	7.6667±0.3333	7.6667±0.6667	9.0000±0.0000	8.3333±0.3333	8.6667±0.6667
10	8.6667±0.3333	4.0000±0.0000	4.0000±0.0000	8.3333±0.6667	8.0000±0.0000	9.0000±0.0000
11	8.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	7.3333±0.3333	4.0000±0.0000

Key: Size of well = 4mm; Org = Organisms; SD = Standard Deviation; Values are mean + SD

Table 7: Effect of temperature on the purified Peptides of Germinated Peas seed

Org	*S. typhi*	*S. aureus*	*Listeria* spp	*E. coli*	*P. aeruginosa*	*B. cereus*
30^{0}C	13.3333±0.6667	13.3333±0.6667	12.6667±0.6667	13.3333±0.6667	12.3333±0.6667	15.3333±0.6667
40^{0}C	11.3333±0.6667	10.6667±0.6667	11.6667±0.3333	10.6667±0.6667	11.0000±0.5773	12.6667±0.6667
50^{0}C	9.3333±0.6667	10.0000±0.0000	8.0000±0.0000	8.0000±0.0000	9.3333±0.6667	8.0000±0.0000
60^{0}C	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000
70^{0}C	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000
80^{0}C	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000
90^{0}C	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000
100^{0}C	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000
121^{0}C	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000	4.0000±0.0000

Key: Size of well = 4mm; Org = Organisms; SD = Standard Deviation; Values are mean + SD

Table 8: Effect of Proteolytic Enzyme Trypsin on fract-ions of Germinated Peas seeds

	$P_{2:4}$		**$P_{4:3}$**		**$P_{6:3}$**		**$P_{8:3}$**	
Trypsin	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
1.0%	4.0000	4.0000	4.0000	4.0000	4.0000	4.0000	4.0000	4.0000
SD	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000
2.0%	4.0000	4.0000	4.0000	4.0000	4.0000	4.0000	4.0000	4.0000
SD	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000	±0.0000
Control	11.3333	12.6667	12.0000	12.6667	11.3333	9.0000	8.6667	11.3333
SD	±0.6667	±0.6667	±1.1546	±0.6667	±0.3333	±0.0000	±0.6667	±0.6667

Key: $P_{2:4}$ = Pea day 2 fraction 4, $P_{4:4}$=Pea day 4 fraction 4, $P_{6:2}$=Pea day 6 fraction 2 and $P_{8:3}$=Pea day 8 fraction SD = Standard Deviation; values are mean + SD, size of well = 4mm. Control= Sodium Benzoate at 1% concentration

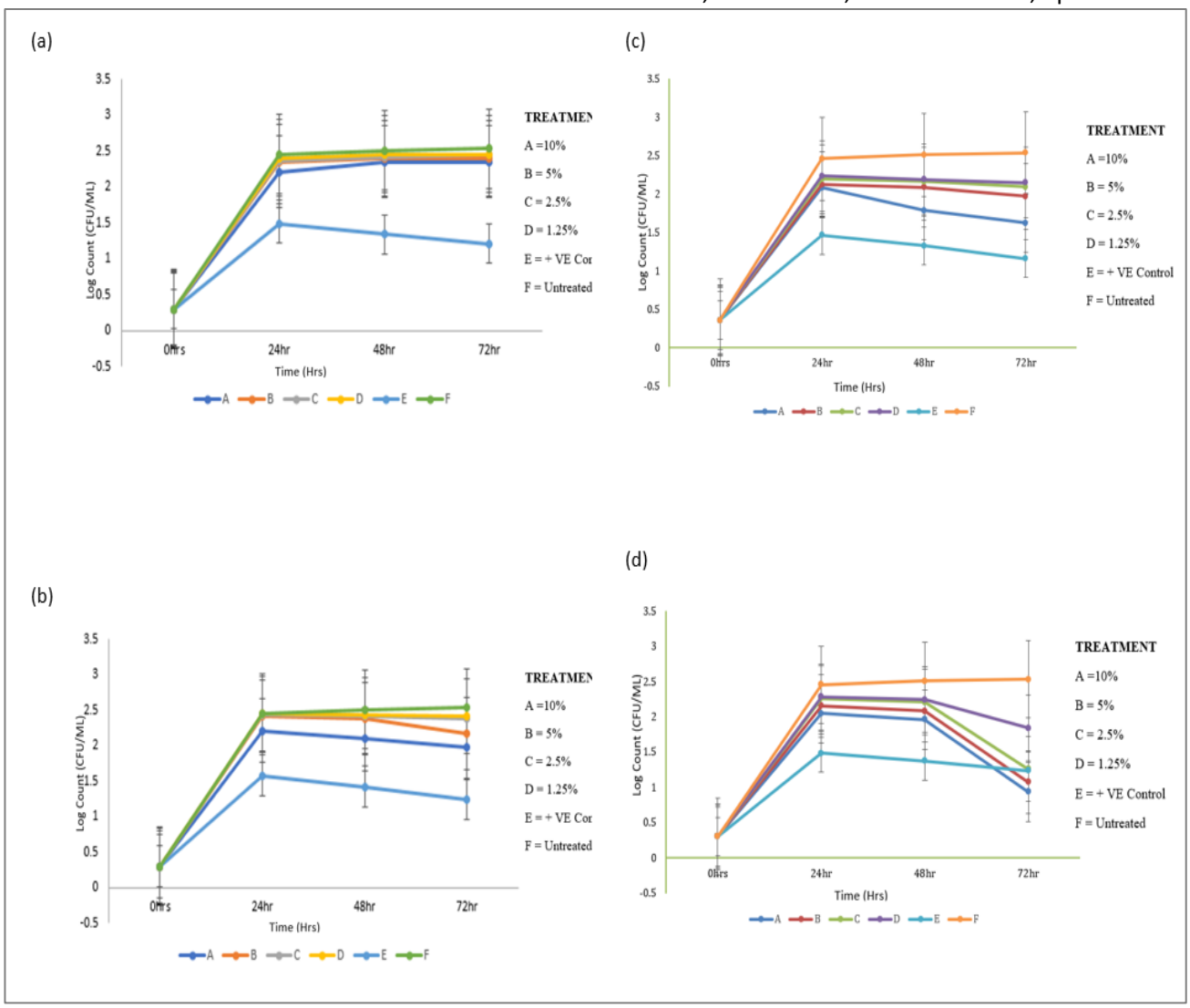

Figure 4: a, b, c, d shows the sanitizing activity of purified Peptides of germinated peas seeds at pH 2.3, at 0hr, 24hr, 48hr and 72 hours respectively. Key: A = 10% Purified peptide, B = 5% Purified peptide, C = 2.5% Purified peptide, D = 1.25% Purified peptide, E = 0.5% Sodium benzoate, F = Untreated

Table 9: Mean Score and Percentage Acceptability of "Zobo drink" treated with purified Peptides of Germinated Peas seeds day two, fraction four ($P_{2:4}$) at pH 2.3

Time (hrs)	A	B	C	D	E	F
0	8.5± 0.167 (94.44%)	8.7±0.153 (96.66%)	8.7±0.153 (96.66%)	8.5± 0.167 (94.44%)	8.5± 0.167 (94.44%)	8.3±0.153 (92.22%)
24	7.9±0.233 (87.77%)	8.0±0.000 (88.89%)	7.9±0.100 (87.77%)	7.9±0.233 (87.77%)	7.8±0.133 (86.67%)	7.2± 0.133 (80.00%)
48	2.6± 0.163 (28.80%)	3.9± 0.276 (43.33%)	3.9± 0.100 (43.33%)	3.7± 0.152 (41.11%)	6.4± 0.163 (71.11%)	5.0± 0.000 (55.55%)
72	1.4± 0.163 (15.55%)	1.2± 0.133 (13.33%)	1.0± 0.305 (11.11%)	1.9± 0.100 (21.11%)	5.1± 0.100 (56.66%)	3.0± 0.000 (33.33%)

Key: A =Treatment with 10%, B = Treatment with 5%, C = 2.5% D = 1.25%, E = Treatment with 0.5% Sodium Benzoate and F= Untreated. SD = Standard Deviation. Values are mean + SD Values are means of sensory scores, while values in ( ) are percentage acceptability scores.

Table 10: Mean Score and Percentage Acceptability of "Zobo drink" treated with purified Peptides of Germinated Peas seeds day four, fraction four ($P_{4:4}$) at pH 2.3

Time (hrs)	A	B	C	D	E	F
0	8.0± 0.211 (88.88%)	8.7±0.213 (96.66%)	9.0±0.000 (100%)	9.0±0.000 (100%)	9.0±0.000 (100%)	8.3±0.153 (92.22%)

To be continued next page

Table 10 Continued

Time (hrs)	A	B	C	D	E	F
24	8.6±0.163 (95.55%)	8.3±0.152 (92.22%)	8.3±0.152 (92.22%)	8.3±0.152 (92.22%)	8.3±0.152 (92.22%)	7.2± 0.133 (80.00%)
48	7.6± 0.163 (84.44%)	7.1± 0.233 (78.88%)	6.6± 0.266 (73.33%)	5.7± 0.152 (63.33%)	7.2± 0.133 (80.00%)	4.5± 0.166 (50.00%)
72	6.5± 0.166 (72.22%)	5.6± 0.221 (62.22%)	5.6± 0.221 (62.22%)	5.3± 0.153 (58.88%)	5.7± 0.153 (63.33%)	3.4± 0.163 (37.77%)

Key: A =Treatment with 10%, B = Treatment with 5%, C = 2.5% D = 1.25%, E = Treatment with 0.5% Sodium Benzoate and F= Untreated. SD = Standard Deviation. Values are mean + SD Values are means of sensory scores, while values in ( ) are percentage acceptability.

DISCUSSION

The maximum germination rate of 91% was obtained with seeds of *Pisum sativum* was obtained at the end of the 8th germination day. This result is in agreement with many researchers who reported that when seeds are placed in an environment favourable to germination, the rate of metabolism is markedly accelerated (Sanchez *et al.,* 2005) (Figure 1).

Protein extraction and purification were essentially carried out on the germinated milled seeds of *Pisum sativum* and revealed varying protein concentrations. This is in line with the works of Saad *et al.* (2010), who reported that plants store proteins in seeds, and in germinating seeds, special endopeptidases trigger storage protein breakdown. Germinating seeds contain abundantly small proteins and peptides (Figure 2), and these peptides have shown strong antimicrobial activity against a number of bacteria. In a similar study by Jabeen and Khanum (2014) using a different seed (*Momordica charantia*), peptides were isolated and purified using similar methods of which fractions were obtained, and three (3) were found to have moderate to high antimicrobial activity (Bera *et al.*, 2023). Peptide purification, achieved by Ammonium Sulphate Precipitation at 75% saturation and dialysis, is in line with the works of Shazia *et al.* (2012) and Bhavith *et al.* (2014), who used the same method to partially purify the peptides of germinated and ungerminated seeds. The results from the SDS-PAGE revealed several bands of protein. However, only those with lower molecular weight were chosen as the target of this study is small peptides which are known to be consistent with existing literature on Plant Antimicrobial Peptides (AMPs) (Figure 3). In another study carried out by Diz *et al.* (2006) antimicrobial peptide of approximately 10kDa was isolated from chili pepper seeds, while Hou *et al.* (2007) purified an antimicrobial peptide with a molecular weight of approximately 8.0kDa. Finally, Jabeen and Khanum (2014) isolated a peptide with a molecular weight of approximately 10kDa from seeds of *Momordica charantia.*

All these studies, including the present study where the isolated peptides were found within this range, reveal that the antimicrobial peptide's mechanism of action is tied to their sequence, size and cationic nature, which governs their interactions with target cells. Furthermore, the lack of antifungal activity of the peptides could possibly be linked to the type of peptides obtained in this study. Generally speaking, Defensins have been found to have high antifungal activity, which differs from antibacterial activity, as reported by Hancock and Chapple, 1999). Additionally, most Defensins are known to have an approximately 5kDa molecular weight (Zhu *et al.*, 2005), and in this study, no peptide with that MW was isolated. Analysis of potential pathogens and spoilage organisms isolated from stale foods and beverages reveals that a significant proportion of these microorganisms are prominent contaminants commonly associated with various types of food and drink products. These organisms, which include both pathogenic bacteria and spoilage microbes, are frequently encountered in environments where food and drink handling is conducted, such as processing facilities and storage areas. The ubiquity of these contaminants in such settings underscores their potential to cross-contaminate entire production batches, often without immediate detection due to the lack of visible signs of contamination. The presence of these microorganisms poses substantial public health risks, as they can lead to foodborne illnesses, including severe gastrointestinal infections and systemic diseases. In extreme cases, exposure to pathogenic strains could result in life-threatening conditions or even fatalities, as reported in a study conducted by Mailafia *et al.* (2017) (Table 2). The antimicrobial activity of the purified peptides shows various levels of activity, from high and low to inactivity. This is in line with the studies conducted by Rehman and Khanum (2011) and Jabeen and Khanum (2014), who found *S. aureus* and *S. typhi* to have the highest antimicrobial activity and that eluted fractions have shown better or higher activity compared to the crude extracts of seeds, thereby indicating that the proteins/peptides isolated from the crude extracts have been purified (Table 4). Antifungal activity was not observed in the two species of mould (*A. niger* and *A. fumigatus*) and two species of yeast (*Candida* spp and *Saccharomyces cerevisiae*). This is not in agreement with a study conducted by Wang and Ng (2007), in which antifungal peptides were isolated with a molecular weight of 7.3kDa from a different species of beans, namely *Phaseolus vulgaris* cv. 'Spotted beans'. This could be a result of the difference in extraction methods in antifungal peptides in both studies, where a procedure that has been proven to work for leguminous defensins like the Reversed-Phased High-Performance Liquid Chromatography (R-PHPLC) was used as described by Wang and Ng, (2000); Wang and Ng, (2002).

The temperatures of 30^{0}C and 40^{0}C were found to have the highest zones of inhibition, and these results show that the peptides of *Pisum sativum* are more effective at moderate temperatures and probably lower temperatures, in line with the findings of Jabeen and Khanum (2014) who also reported similar findings with peptides of *Pisum sativum* seeds (Table 6). The result from the effect of pH on the peptides indicated that the activities of the purified peptides are pH-dependent and vary for different microbes. The results further revealed that the purified peptides are stable between a narrow range of pH 4-7, which is similar to the findings of Rehma and Khanum (2011) for peptides purified from *P. sativum* (Table 7). This finding could also be tied to the reason for the peptide's ability to sanitize the *Zobo* drink as well as extend its shelf life. The proteinaceous nature of isolated peptides of *Pisum sativum* was confirmed with the use of proteolytic enzyme trypsin against *S. aureus* and *E. coli* (Table 8). The effect of sodium dodecyl sulfate (SDS) at different concentrations in this research shows that at low concentrations of SDS, activity is decreased, while at high concentrations of 2.0% and 5.0%, activity was completely lost. This is similar to the findings of Kaktchman *et al.* (2012) who reported reduced activity of peptides by SDS. The result of the sanitization activity of the laboratory-prepared 'zobo' drink at Ph 2.3 carried out at 0hr, 24hr, 48hr and 72hrs using the extracted peptides of *Pisum sativum* ($P_{2:4}$, $P_{4:4}$, $P_{6:2}$, $P_{8:3}$). The APC converted to log count were found to have increased after 24hrs in all the treatment A – F including treatment E which contains a known conventional food preservative (0.5% sodium benzoate). By the 2nd day of storage (48hrs), the log count was found to have significantly decreased except for treatment F (untreated zobo drink), which served as the negative control, and also the peptide of germinated peas day 2 fraction 4 ($P_{2:4}$) which recorded a *log* increase in count. This is in line with the findings of Bukar *et al.* (2010) (Figure 4).

Similarly, the variation in pH, temperature and titratable acidity shows that after the addition of the various concentrations of the peptides, there was a significant increase in the pH of the zobo drink at 0hr, particularly with treatment A (10% concentration). Furthermore, the titratable acidity of treatment A (10% concentration) and E (0.5% sodium benzoate) were found not to be significantly different, while there was a slight variation in temperature across the treatments in line with studies by Doughari (2007); Bukar (2012). After 24hrs, 48hrs and 72hrs of storage, there was a further decrease in the pH temperature while the titratable acidity increased. This is in line with the findings of Ndidi *et al.* (2012), who reported that the titratable acidity of zobo drink (ambient and refrigerated) showed an increasing trend with increased storage. The sensory evaluation was carried out for up to 72 hours of storage at room temperature. From the results, the sensory evaluation revealed that the shelf life of the *zobo* drink was extended up to 72 hours with a high percentage likeness of 72.22%. The best-performing peptide was $P_{4:4}$ (Germinated peas seed day four fraction 4) at the highest concentration of 10%) which was well above the positive control (treatment E, 0.5% sodium benzoate) (Table 9). The least performing treatment was $P_{2:4}$ (Germinated pea seed day two fraction four) with 15.55% likeness even at the highest concentration of 10% (Table 10). General analysis of variance (One-way ANOVA) on the percentage acceptability of the "*zobo*" drink using different treatments A – F shows that there is a statistically significant difference between the treatments at 5% level of significance.

CONCLUSION

In conclusion, the study on the preservative properties of peptides derived from *Pisum sativum* in the 'Zobo' drink highlights the potential of these natural compounds as effective biopreservatives. The peptides exhibited significant antimicrobial activity against various spoilage and pathogenic microorganisms, thereby extending the shelf life of the beverage. Additionally, their ability to inhibit oxidative processes and maintain the sensory attributes of 'Zobo' drink further enhances their value as preservatives. This research opens up new possibilities for utilizing plant-derived peptides as safer and sustainable alternatives to synthetic preservatives in the food and beverage industry. Furthermore, future research should prioritize the development of advanced methodologies for optimizing both the extraction efficiency and molecular stability of these peptides. This should involve refining extraction methods to maximize yield and purity while simultaneously addressing factors such as thermal degradation, oxidation, and enzymatic activity that may compromise peptide integrity. Lastly, a comprehensive evaluation of these peptides under various industrial processing conditions, including pH fluctuations, temperature variations, and prolonged storage, is essential to ensure their sustained bioactivity and efficacy in large-scale beverage preservation applications.

RECOMMENDATIONS

1. There is a need for further research to determine the molecular structures, amino acid sequence and even the identity of the peptides isolated from the germinating seeds of *Pisum sativum*.

2. There is a need to further test the preservative properties of the peptides on other minimally and fully processed foods.

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