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BACTERIAL FLORA AND SENSORY CHARACTERISTICS OF FRESH AND SALTED *VERNONIA AMYGDALINA* LEAVES

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ABSTRACT
In this study, the microbial flora and sensory characteristics of fresh and salted *Vernonia amygdalina* leaves were investigated. The leaves were subjected to light salt, light brine, light brine and vinegar and heavy salt treatments for two weeks. Compared with the fresh leaves, all treatments gave leaves with lower pH and bacterial counts. Heavy-salted and light brine and vinegar treated leaves had the lowest pH and bacterial counts, and gave products containing *Lactobacillus* and *Streptococcus,* but devoid of *Staphylococcus spp*. In addition, they gave leaves with the best organoleptic characteristics, indicating that they were the most effective. The use of salting as a process for the development of value-added products from *V. amygalina* leaves is discussed.

KEYWORDS: *Vernonia amygdalina* leaves, salting, bacterial counts, microbial flora, probiotics

INTRODUCTION

Using simple technologies, it is possible to alter some nutritional and organoleptic characteristics of fruit and vegetable matrices by modifying them in a controlled way, such as salting and pH modification (Jones and Etchells, 1944; Battock and Azam-Ali, 1998). Their content of beneficial nutrients, such as minerals and vitamins makes them ideal substrates for the growth of desirable bacteria, which by fermentation would in turn, elaborate flavour compounds, decompose antinutrients and increase the availability of nutrients, and improve their palatability (Teucher *et al*., 2004; Gibson *et al.*, 2006). The salting or brining of vegetables offers tremendous possibilities both for their commercial and home preservation, and enrichment (Axtell *et al*., 2008; Oboh and Madojemu, 2010; Oboh *et al*, 2013, Oboh and Madojemu, 2016). In the process the salt exerts a selective action on the naturally occurring organisms to promote a desirable fermentation. Salt tolerant microorganisms use as their nutritive material, the soluble constituents that diffuse out of the vegetable as a result of the action of the salt on vegetable tissue. These fermentative organisms bring about the production of various compounds, principally lactic acid, but also acetic acid, alcohols, and considerable amounts of gas. The production of a sufficient amount of acid makes the medium unsuitable for the growth of food spoilage bacteria. In addition, the acid and other microbial metabolites alter the flavour of the food (Battock and Azam-Ali, 1998; Galgano *et al.*, 2015). Compounds (prebiotics) and organisms (probiotics) in fermented foods can cause desirable changes in the composition and/or activity of the gastrointestinal microbiota resulting in health benefits (Perdigon *et al*., 1987; FAO, 2007; Galgano *et al.*, 2015).

Vernonia amygdalina Del., variously known as bitter leaf (English), oriwo (Edo), ewuro (Yoruba), shikawa (Hausa), and olubu (Igbo), is a tropical shrub, 1-3 m in height with petiole and leaf of about 6 mm in diameter, and elliptic in shape (Igile *et al*., 1995). The leaves are dark green in colour, with a characteristic smell and a bitter taste. The species is indigenous to tropical Africa where it is found wild or cultivated (Bosch *et al*., 2005). The primary use of the plant is as a source of green leafy vegetable for culinary application. The leaves are eaten, after crushing and washing thoroughly to remove the bitterness (Mayhew and Penny, 1998). It has various secondary uses – in folk medicine (all parts of the plant), as an ornamental, for forage (the leaves), for wood fuel (stems and branches) (Mayhew and Penny, 1998; Bosch *et al*., 2005). Medicinal applications of the plant include the use of the leaf extract as a laxative (Gill, 1992; Awe *et al.*, 1999) antihelmitic and antimalarial (Abosi and Raseroka, 2003; Iwalokun, 2008). The antithrombotic and hypoglycemic effects of the leaf extract, and its hypolipidaemic effect in diabetic-hyperlipidaemic (Akah and Okafor, 1992; Nwanjo, 2005) and normoglycaemic-hyperlipidaemic rats (Adaramoye *et al.*, 2008; Oboh and Enobhayisobo, 2009) have been reported. The leaf extract also exhibits antimicrobial (Akinpelu, 1999; Oboh and Masodje, 2009; Mboto *et al.*, 2009) and anti-tumourigenic properties (Izevbigie *et al.*, 2004).

Other studies have been undertaken in order to increase knowledge of the composition and utilisation of *V. amygdalina* leaves in food and nutrition. These include nutrient composition and antimicrobial activity (Oboh and Masodje, 2009), the effect of salting and drying on the organoleptic characteristics and nutrient composition (Oboh and Madojemu, 2010), the effect of salting on the phytochemicals, fungal flora and nutrient composition (Oboh *et al*., 2013), and the fractionation of *V. amygdalina* and analysis of the leaf fractions (Oboh *et al*., 2016). It was found that *V. amygdalina* leaves are rich in minerals, vitamins and phytochemicals, and contain modest amounts of proteins and lipids, all of which are moderately or highly retained in the dried and salted products (Oboh and Madojemu, 2010; Oboh *et al*., 2013), and the fibrous and protein fractions of the leaves (Oboh *et al*., 2016). Fructo- and galacto-oligosaccharide prebiotics were found in the aqueous extract, and prebiotic effects of this extract on the gastrointestinal tract of an animal model were observed, including protection of the animals against some pathogens (Ezeonu *at al*., 2013, 2016).

This study was undertaken to investigate the effects of salting and brining on the bacterial flora of *V. amygdalina* leaves. The leaves were subjected to four treatments – heavy salting, light salting, light brining, and light brine and vinegar treatment for 14 days, and their bacterial flora and sensory characteristics were examined. The use of salting for the preservation, as well as the development of novel food products from the leaves is discussed.

MATERIALS AND METHODS

Materials

Vernonia amygdalina Leaves

V. amygdalina leaves were harvested from the vegetable garden of the Faculty of Agriculture and Agricultural Technology, from stands identified by staff of the Department of Crop Science of the faculty. Whole leaves were used for salt treatments.

Reagents

Sodium chloride and nutrient agar were from Merck, Darmstadt, Germany. White vinegar (5% acidity) was from Magic Time, Doral, Florida, USA.

Methods

To investigate the effect of salting on the bacterial flora of *V. amygdalina* leaves, a study was conducted using the following treatments:

Heavy Salting

V. amygdalina leaves were gently rinsed to remove dirt, and the water drained. Salt (37.5 g) and leaves (150.0 g) were mixed well and filled into a plastic bucket. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed on it. Brine made of salt (37.5 g) dissolved in water (150.0 ml) was added until the pressure plate was slightly submerged. The buckets were stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003), at ambient temperature of 31.0-32.5^{o}C.

Light Salting

Rinsed and drained *V. amygdalina* leaves (150.0 g) were mixed well with dry salt (3.75 g), filled into a plastic bucket and packed tightly. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003), at ambient temperature of 31.0-32.5^{o}C.

Light Brining

To rinsed and drained *V. amygdalina* leaves (150.0 g) was added light brine (3.75 g salt dissolved in 150ml water). The brine was added to the vegetable in layers (i.e.by starting with a layer of vegetables and the addition of brine, followed by another layer of vegetables and addition of more brine, and so on) in a plastic bucket, packing tightly. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003), at ambient temperature of 31.0-32.5^{o}C.

Light Brine +Vinegar

Light brine and vinegar was made by mixing salt (7.5 g), vinegar (7.5 ml) and water (150.0 ml). This was added to washed and drained *V. amygdalina* leaves (150.0 g) as described above for light brining. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed on it. The set up was stored in a cool dry and shaded place for two weeks, at ambient temperature of 31.0-32.5ºC.

Sensory Evaluation

A sensory evaluation of the fresh and salted leaves was undertaken as follows (IFT, 1981). A description of colour, taste, odour, and texture of the formulations, based on sensory perception was carried out by an untrained panel of six judges (3 male and 3 female) selected from the final year biochemistry class of Benson Idahosa University, who apparently had no defect in their ability to perceive the characteristics examined. Labelled samples of the fresh and salted leaves were placed in glass petri dishes on a table covered with white cardboard which was placed in a well- lit and ventilated room. Prior to inspection of samples panellists were provided with a sheet containing the following descriptions of characteristics and were asked to record those which were closest to their observations (Table 1).

Table 1: Descriptive sensory evaluation sheet

Colour	Yellow-green	Lemon-green	Light green	Dull dark green	Bright dark green
Taste	Mildly salty and slightly bitter	Mildly salty and bitter	Salty, bitter and tangy	Very salty, very bitter, and tangy	Very bitter and very salty
Smell	Rotten, very offensive	Hydrogen sulphide-like, offensive	Mildly hydrogen sulphide –like, not offensive	Faint, not offensive smell.	Fresh, leafy, aromatic; attractive.
Texture	Degraded and in pieces, slimy	Limp, with smooth surface	Less firm than the fresh leaves, with a less rough surface	Less firm than the fresh leaves; rough surface	Firm, with rough surface

Examination of samples was undertaken by one panellist at a time. Subsequent open discussion by the panellists of their observations (moderated by a leader chosen by the group) resulted in the final description of the fresh and salted leaves.

Analytical Procedure

The pH of the distilled and deionized water extract of the vegetable was measured at an ambient temperature of 27.0ºC using a previously calibrated pH meter (Jenway Model 3505, Camlab, UK).

Microbiological Analysis

Total Viable Microbial Counts

These were estimated using the pour plate method. A ten-fold serial dilution of samples was made (i.e. 1 ml of the fresh or treated *V. amygdalina* leaf extract was made up to 10 ml with sterile distilled water in test tubes). Enumeration of microorganisms in the extract was done by inoculating 1ml aliquots of the diluted sample into labelled Petri dishes followed by pouring of the nutrient agar. The serial dilutions of the extracts were done up to 10^{-2}. The plates were allowed to solidify, inverted, and then incubated at an ambient temperature of 28.0ºC for 24 hours after which the colonies were counted (Cheesebrough, 2000).

Isolation of Microorganisms

Discrete bacteria colonies were isolated from nutrient agar plates and further sub-culturing on nutrient agar plates was carried out in order to obtain pure colonies; these were inoculated and stored in slants at 4.0°C for further laboratory analysis (Cheesebrough, 2000).

Identification of Microbial Isolates

Pure bacteria isolates were identified using morphological (microscopic) and biochemical characteristics as described by Cheesebrough (2000).

RESULTS AND DISCUSSION

The acidity of foods is very important because it influences the kind of spoilage that may occur and hence the way that foods are processed. Most bacteria grow readily at a pH near 7, when a food is neutral. Their growth is more easily inhibited at lower pH of about 4 to 6 (Eyabi, 2001; Rose, 1975). Table 2 shows the pH values of *V. amygdalina* leaves subjected to different salting procedures.

Table 2: Effect of salting on the pH of *V. amygdalina* leaves

Leaves	***V. amygdalina***
Fresh (day 1)	5.83
Treatments	
Light brining	5.70
Light salting	5.65
Heavy salting	5.43
Light brine + vinegar	5.09

The fresh *V. amygdalina* leaves had a pH of 5.83. This decreased to 5.70 for the light brined, 5.65 for the light salted, 5.43 for the heavy salted, and 5.09 for the light brine and vinegar treated leaves. Treatment with the combination of salt and vinegar resulted in the highest decrease, followed by treatment with concentrated salt solution (i.e. heavy salting). The decrease in the pH of the leaves indicates increase in acidity resulting from fermentation and/or addition of vinegar. Thus, salting created the right environment for the growth of acid-producing bacteria.

The effect of preservation treatments on the bacterial count of *V. amygdalina* leaves is shown in Table 3.

Table 3: Effect of salting on the bacterial count of *V. amygdalina* leaves

Leaves	**Bacterial count (cfu/ml)**	**% Decrease[b] (relative to bacterial count on day 3)**
Fresh (day 1)	0	-
On day 3	40×10^2	-
Treatments[a]		
Heavy salting	10×10^2	75.0
Light salting + vinegar	8×10^2	80.0
Light brining	16×10^2	60.0
Light salting	19×10^2	52.5

[a] Treatments of fresh leaves were for 14 days at the end of which bacterial counts were taken.
[b] %Decrease = 100 – (bacterial count of the treated leaves /bacterial count of untreated leaves on day 3 x 100).

Freshly harvested leaves were bacteria-free, due to their antibacterial activity (Oboh and Masodje, 2009). This activity diminished due to postmortem loss of physiological activity and subsequent breakdown of plant tissue resulting in loss of resistance to bacterial infection. Bacterial count of untreated *V. amygdalina* leaves on the third day was 40 x 10^2 cfu/ml. Compared with the 3-day old untreated leaves, the treatments decreased the microbial load. The highest decrease (80.0%) resulted from light brine + vinegar treatment, followed by heavy salting, which resulted in a 75.0% decrease. The other treatments were less effective, resulting in 60% and 52.2% decrease for light brining and light salting respectively.

Bacterial genera associated with fresh and salted *V. amygdalina* leaves are shown in Table 4.

Table 4: Bacterial genera observed in fresh and salted *V. amygdalina* leaves

Leaves	***Streptococcus spp.***	***Staphylococcus spp.***	***Lactobacillus spp.***
Fresh	-[a]	-	-
On day 3	+[b]	+	-
Treatments			
Heavy salting	+	-	+
Light brine and vinegar	+	-	+
Light brining	-	+	+
Light salting	-	+	+

[a]-: Absent. [b]+: Present

The fresh leaves yielded no bacterial growth. In the absence of any preservation treatment, strains of *Streptococcus* and *Staphylococcus* were observed on the third day. Only *Streptococcus* and *Lactobacillus* strains were observed in leaves preserved in concentrated salt solution and light brine + vinegar, indicating that these treatments ensured a desirable microbial succession, by creating an environment suitable for the growth of these organisms but unfavourable for the growth of *Staphylococcus. Lactobacillus* strains that produce bacteriocin active against *E. coli, Staphylococcus aureus, and Bacillus cereus* have been isolated from carrots (Joshi *et al.*, 2006), and the absence of *Staphylococcus* in the products of these treatments was probably due to the lower pH (relative to the fresh leaves), the high salt concentration (of the heavy-salted leaves), and/or the presence of bacteriocin. However, the presence of bacteriocins was not examined in this study. *Lactobacillus* and *Staphylococcus* were present in the light brine and light salt treated leaves. These treatments were however, unsuitable for the growth of *Streptococcus.* Strains of *Staphylococcus* are pathogenic and are implicated in food spoilage. Compared with the products of heavy salting and light brine and vinegar treatment, the absence of the acid-producing *Streptococcus* resulted in higher pH (Table 2) and the presence of

Staphylococcus indicated poor preservation of the leaves (Table 5).

Previous authors (Oboh *et al*., 2013) have reported the presence of toxigenic fungi in fresh and salted *V. amygdalina* leaves. *Aspergillus flavus* was found in fresh and salted leaves. *Fusarium*, which was not detected in the fresh leaves, was found in the light brine and vinegar treated samples. Aflatoxin was detected in both fresh and preserved leaves; compared with the former, preservation in concentrated salt solution caused a 93.33% decrease, and in light brine and vinegar, a 64.17% decrease in the fungal count of the leaves (Oboh *et al*., 2013). Thus apart from the elimination of *Staphylococcus*, decrease in total bacterial count, and selection for *Lactobacillus* and *Streptococcus* observed in this study, both treatments may also cause a considerable reduction in total (including toxigenic) fungal count.

Probiotics are considered beneficial when they form part of the diet of humans (Saavedra *et al*., 1994; Sloan, 2004; Granato *et al*., 2010; Galgano *et al*., 2015). Microbes are used as probiotics including bacteria, yeast, and mould. The genera and species that have been used are *Lactobacillus, Streptococcus, Leuconostocc, Pediococcus, Propionibacterium, Enterococcus, Bifidobacterium, Bacillus, Saccharomyces cerevisiae, Candida pintolopessi, Aspergillus niger and Aspergillus oryzae* (Galgano *et al*., 2015). Lactic acid bacteria are considered a major group of probiotic bacteria and are commonly used in both humans and animals (Soccol *et al*., 2010). The dairy sector, which is strongly linked to probiotics, is a large functional food market, and about 78% of current probiotic sales in the world are delivered through yoghurt. However, an increased demand for non-dairy probiotic products comes from the ongoing trend of vegetarianism and possible adverse health effect of consumption of dairy products due to milk cholesterol content, and lactose intolerance (Granato, 2010). The bacterial succession which resulted in the appearance of *Lactobacillus* and *Streptococcus* suggests that heavy salt and light brine and vinegar treatments of *V. amygdalina* leaves could produce a non-dairy matrix suitable for the growth of probiotic strains of these organisms.

The production of acid (indicated by decrease in pH), caused the decrease in microbial count (compared with the fresh leaves) by making the medium unsuitable for the growth of food spoilage bacteria. Added acid, in the form of vinegar also contributed to the antibacterial effect of light salt and vinegar treatment, which though of low salt concentration, gave the product with the lowest pH. Previous authors (Oboh and Madojemu, 2010, Oboh *et al*., 2013) observed an osmotic effect on the leaves by the high salt concentration employed in the heavy-salting process, which caused a decrease in their moisture content. Thus for the heavy-salted leaves, the presence of acid in the medium (produced by the halophilic organisms present), the considerable dehydration of the leaves, and the salinity of the medium, acted in a cooperative manner to prevent the growth of spoilage organisms, and ensure the preservation of the leaves.

The acid and other microbial metabolites produced as a result of the treatments altered the flavour of the leaves. The sensory characteristics of fresh and fermented *V. amygdalina* leaves (Table 5) were similar to those observed in previous studies (Oboh and Madojemu, 2010, Oboh *et al*., 2013). The heavy-salted and light brine and vinegar treated leaves were the closest in appearance to the fresh leaves. The latter smelled like *ogiri*, a condiment made in Nigeria by the fermentation of melon (*Citrulus lanatus*) seeds (Achi, 2005). They were slightly salty, with a mildly bitter taste, with a hint of tanginess. These characteristics indicate that they would be suitable for inclusion in salads. Heavy-salted leaves had a very salty and very bitter taste; except for their salty taste, they resembled the fresh leaves. In culinary applications, their high salt content would determine the extent of their utilisation. However, *Aspergillus flavus* has been detected in both heavy salted and light brine and vinegar treated leaves, and also *Fusarium* in the latter (Oboh et al, 2013). Thus the level of aflatoxin in the leaves from both treatments and of fumonisin in the light brine and vinegar treated leaves must be determined and found to be safe before they can be recommended for use as food. The light salt and light brine treatments gave less salty, partially debittered leaves, but because of their contamination with *Staphylococcus* they are unsuitable for food application.

Table 5. Characteristics of fresh and salted *V. amygdalina* leaves

			Treatments		
Characteristics	**Fresh**	**Light brine**	**Light salt**	**Light brine + vinegar**	**Heavy salt**
Colour	Dark green	Dull dark green	Dull dark green	Dull dark green	Dark green
Taste	Very bitter	Slightly salty and slightly bitter.	Slightly salty and slightly bitter.	Slightly salty; bitter with a hint of tanginess	Very bitter and very salty.
Smell	Fresh leafy, aromatic smell	Slight but not offensive odour. Smells like *ogiri.*	Slight but not offensive odour. Smells like *ogiri.*	Slight but not offensive odour.	Fresh leafy aromatic smell.
Texture	Firm, with slightly rough surface	Less firm and rough than the fresh leaves.	Slightly more firm than product of light brining. Less rough than the fresh leaves.	Retained more of the original structure of the fresh leaves than products from light salt, or light brine treatment. Smooth feel.	Similar to the fresh leaves- firm and slightly rough leaf surface.

CONCLUSION

The results of this study indicate that heavy salting, and light salt and vinegar treatments of *V. amygdalina* leaves offer several benefits- their preservation, development of flavour, and the possibility of their use as a matrix for the growth of probiotics. These benefits offer opportunities for product and process development, resulting in the addition of value to the produce.

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