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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Effect of wheat flour substitution, maize variety and fermentation time on the characteristics of *Akara*, a deep oil fried dough product

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An investigation into the possibility of using local cereal resources (maize) to develop composite flour suitable for the production *of Akara* (a deep fried fermented dough) was undertaken. The experimental design was a 4 x 5 x 5 factorial experiment. Factor one was the maize variety (CMS 8806, CMS 8501, CMS 9015 and CMS 8704), factor two the substitution level (0, 10, 20, 30 and 40%) of wheat for maize flour and factor three was the fermentation times (0, 30, 60, 90 and 120 min). Dough nuts were produced from composite flour from wheat and maize flour. For the production of maize fine flour, wet milling of de-hulled, soaked grain was made followed by drying and sieving. Composite flours (32.4%) and other ingredients (13.5% sugar, 0.5% instant bakers' yeast and 0.3% iodised salt) were mixed with water, whipped for 10 min and cut into samples of 40 g which were manually shaped into balls. They were allowed to ferment and rise prior to deep frying. Flour samples were analyzed for their physico-chemical and functional properties. A mixed panel of women operating in dough nut production and some regular consumers was trained for sensory evaluation. The sensory attributes considered were colour, taste, texture and overall acceptability on a five point scale. Results showed that maize cultivars did not affect the quality of fine flours from wet milling. In composite flours, proteins, lipids and ash contents were significantly reduced as the level of maize flour increased. An inverse trend was observed with water absorption capacity. Dough swelling capacity and *Akara* density decreased with increase in maize substitution levels independently of the fermentation time and maize variety. Similarly, sensory scores decreased with increases in the substitution level independently of the maize cultivar used for composite flour preparation. However, *Akara* from composite flours had overall acceptability scores between 4.2 and 2.8 on a five-point scale. *Akara* prepared from CMS 8806 and CMS 8704 all of yellow color showed the highest sensory scores. From the results obtained, it could be suggested that composite flour from wheat and maize with up to 40% substitution level is acceptable for local commercial production of *Akara* with preference given to yellow maize.

Key words: Maize, flour, doughnut, characteristics.

INTRODUCTION

Traditional cereal foods play an important role in the diet of the people of Africa, particularly in cereal producing zones. Flour from various cereals is one of the main raw material used in the production of popular food products with high acceptability, good storage characteristics and affordable cost. One of such food product is *Akara*, a deep oil fried fermented dough, produced by women, using simple processing methods. It is a breakfast food

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largely consummed by children and adults in West and Central Africa, especially in Cameroon (Cerdan et al., 2004). Initially, dough for *Akara* was made from 100% imported wheat flour exposing production to price fluctuation due to importation and transportation costs. In an attempt to minimize production costs in rural areas, village women have developed composite flours by incurporating various amounts of rice or maize flour into wheat flour for *Akara* production. This has led to a lot of variability in taste, texture, size and appearance in the *Akara* from one producer to another and from one village to another. Studies conducted to develop composite flour suitable for bread production suggested that bread of acceptable characteristics could be obtained from composite flour with up to 40% substitution (Tiekoura, 1994; Keregero and Mtebe, 1994; Iwuoha et al., 1997). Bread with 40% or less sorghum composite flour had scores of relative acceptance above 70% as compared to the standard 100% wheat bread. From above 40% sorghum composite flour, bread produced received low acceptability scores of 6.9 on a nine-point scale (Carson et al., 2000). With maize flour, incorporation rate of up to 15% was shown to be acceptable suggesting that the type of cereal used affects the optimum acceptable level of flour incurporated. However, these results have had little application among bakers because the relative benefit from maize composite flour does not justify the extract expenses, time and production adjustments needed. Doughnut producers on the other hand are voluntarily adding various amounts of cereal flours in their dough to obtain besides *Akara*, different types of fried dough products *Cen-cen*, *Taara-pott-en, Wardi* and *Wayna* (Lopez and Muchnik, 1997).

The characterization and standardisation of the use of composite flour for doughnuts would strengthen and encourage its production. Based on availability, maize flour is probably the cheapest possible ingredient to be considered. However, the physico-chemical composition of maize grain from various cultivars varies considerably (FAO, 1993; IRA-NCRE, 1990) and this may affect the functionality of resulting maize flour. The method by which flour is produced (Watson, 1984; Munck, 1995) should equally be considered in developing composite flours. This study was designed to evaluate the effects of wheat flour substitution rate on composite flours from maize cultivars on the properties of *Akara*.

MATERIALS AND METHODS

Maize grain and wheat flour sources

Maize (*Zea mays* L.) grains used were obtained from the collection of Cameroon Maize Selection (CMS) of the maize breeding program of the Institute of Agricultural Research for Development (IRAD), Cameroon. Four leading cultivars were considered based on their high adoption by farmers. They included CMS 8501 and CMS 8704 both suitable for high rain fall or humid climate with long maturing cycle, high yielding potentials (5 to 7 ton/ha) and with white and yellow colors, respectively. The other two cultivars suitable to arid climate were CMS 8806 and CMS 9015 with yellow and white colors, respectively, with short maturing cycles and moderate yields of 4 to 5 ton/ha. All these varieties were grown under the same environmental conditions. Commercial baker's wheat flour (12% protein) purchased from the local market was used as a control.

Pre-treatment of maize grains and flour production

Flours from maize were produced in a wet milling process (Ndjouenkeu et al., 1989). De-hulled grains free of bran particles were soaked in clean tap water (1:3 w/v) for 4 h after which they were removed, partially sun dried on a mat for 2 h and milled in a hammer mill. The flour obtained was sun dried for 6 h and fractioned through a 400 µm sieve. Fine fraction of particle diameters less than 400 µ were used to formulate composite flours by thoroughly mixing in a baker mixer.

Experimental design

Composite flours in ratios of 90:10, 80:20, 70:30 and 60:40 w/w for wheat and maize flours, respectively, were reconstituted and used in the production of *Akara*. Whole wheat flour was used as control. A 4 x 5 x 5 factorial experiment was used with four maize varieties (CMS 8806, CMS 8501, CMS 9015 and CMS 8704), five substitution levels (0, 10, 20, 30 and 40% maize) and five fermentation times (0, 30, 60, 90 and 120 min).

Dough samples and doughnut production

Composite flours (32.4%) and other ingredients (13.5% sugar, 0.5% instant bakers' yeast and 0.3% iodised salt) were used for dough preparation. The ingredients were introduced in potable water (53.3 to 65.3%) according to the specific water absorption capacity (WAC) of composite flour, mixed for 3 to 5 min after which appropriate mass of flour was added and kneaded for 2 to 3 min. The dough was whipped for 10 min using a fork mixer (Bonnet, France) and cut into samples of 40 g which were manually shaped into balls. The balls were divided into two groups from which one group was fermented and tested for swelling capacity after 0, 30, 60, 90 and 120 min. The other group was fried in deep oil for 5 min after 90 min of incubation at room temperature (25 to 28°C).

Physico-chemical analysis

The dehulling and milling rates of maize grains were calculated as the ratio of the weight of dehulled grains and milled flour (400 µm) to that of whole and dehullled grains, respectively. Samples of 1000 grains were weighed, and whole wheat and composite flours analysed for moisture, proteins, lipids and ash contents as described by official methods of analysis (AOAC, 1999). Moisture content was determined as weight loss of 10 g sample after drying for 24 h at 102°C. Lipid content was calculated by weight loss after 16 h hexane extraction in a soxhlet apparatus. Protein content was determined by Kjeldahl digestion technique followed by spectrophotometric determination of resulting nitrogen. The ashing was done in a muffle furnace at 550°C for 24 h. Total carbohydrate was obtained by calculation (Egan et al., 1981).

Functional property analysis of flour and dough samples

Water absorption capacity was determined by the method of Phillips (Phillips et al., 1988). A sample of 1 g of the composite orwheat flour was mixed in 10 ml of distilled water and mechanically mixed for 30 min in a KS 10 agitator. The mixture was centrifuged

Table 1. Physico-chemical properties of maize and wheat flours (means ± sd)*.

Property	Flour sources				
	Wheat	CMS 8806	CMS 9015	CMS 8501	CMS 8704
Dehulling yield (%)		72.1±1.5^{c}	70.8±2.0^{a}	71.3±1.1^{b}	72.7±1.5^{c}
Flour yield (%)		57.3±1.2^{b}	63.3±2.1^{c}	57.1±1.1^{b}	49.2±1.9^{a}
1000 Grain weight (g)		188.4±0.9^{a}	287.6±2.2^{c}	305.8±3.1^{d}	271.7±0.4^{b}
Carbohydrates (%MS)	70.4±0.1^{a}	78.5±0.3^{b}	79.1±0.1^{bc}	78.6±0.3^{b}	78.4±0.2^{b}
Moisture (%MS)	12.9±0.1^{a}	13.9±0.0^{c}	13.5± 0.4^{b}	14.1± 0.1^{c}	13.1±0.0^{b}
Proteins (%MS)	12.0±0.1^{b}	4.1±0.3^{a}	4.6±1.0^{a}	4.1±0.1^{a}	4.7±0.4^{a}
Lipids (%MS)	3.8±0.1^{c}	2.5±0.2^{a}	2.4±0.0^{a}	2.7±0.1^{a}	3.4±0.1^{b}
Ash (%MS)	0.8±0.0^{c}	0.7±0.0^{b}	0.5±0.0^{a}	0.3±0.0^{a}	0.4±0.1^{a}

Means in the same line with different letters are significantly ($p<0.05$) different.

at 4500 rpm for 30 min on a desktop centrifuge (Bioblock Scientific MLWT.62.1). The resulting sediment (M_2) was weighed and then dried at 105°C for 24 h and the dry weight (M_1) was determined. The WAC was then calculated as follows:

$$WAC\ (\%) = 100 \times (M_2-M_1)/M_1$$

Dough swelling capacity (DSC) was determined from the relative swelling (Delhaye et al., 1984) of 40 g dough sample gently filled to mark in a 10 cm graduated cylinder. After incubation at 30°C for the desire fermentation time, the DSC was estimated as the ratio of the height of each dough sample to that of wheat multiplied by 100.

Physical and sensory analysis

Physical parameters (weight and diameter) of fried doughnut were measured. Sensory evaluation was conducted using the quantitative descriptive analysis (Powers, 1984) by a 12 member mixed panel. Panelists were selected based on their past habit of consuming the conventional wheat *Akara.* They underwent training sessions on how to evaluate the organoleptic characteristics of *Makala* samples. Sensory attributes considred were flavor, color, taste, firmness and overall acceptability. The intensity of each attribute was scored on levels of likeness ranging from 1 (extreme dislike) to 5 (like extremely). Coded doughnut samples were presented to the panelists in disposable plates without any additional ingredient. Water was provided for rinsing the mouth between samples.

Statistical analysis

All measurements were carried out in triplicate and data obtained were treated with the statistical package SPSS (1993). The analysis of variance (ANOVA) was used to test the effects of the factors on the properties measured and the Duncan's multiple range test was used to separate treatment means whenever there were significant differences.

RESULTS

Flour physico-chemical characteristics

The physico-chemical composition of maize and wheat flours is presented in Table 1. The maize cultivars used were characterised by specific distinct 1000 grain weight, dehulling and milling rates. However, their fine flour fractions exhibited similar composition as far as protein, carbohydrate and moisture contents are concerned. The incorporation of various levels of these maize flours into wheat flour significantly affected the chemical properties of resulting composite flours (Figures 1 and 2).

Protein, lipids and ash contents significantly ($p<0.05$) decreased as the level of maize flour substitution increased independently of the cultivars used. The high lipid content of the grains of maize cultivar CMS 8704 led to composite flour with highest fat contents. Similar results where observed in *bro* (maize composite bread) made with blend of wheat and maize flours in the ratio of 60:40, respectively (Tiekoura, 1994).

Water absorption capacity

The maize cultivars used affected WAC of composite flours (Figure 3) with the yellow cultivars CMS 8806 showing the highest ($p<0.05$) values of WAC followed by CMS 8704. Wheat flour samples showed significantly lower ($p<0.05$) WAC values despite their higher protein content. WAC of composite flour increased significantly ($P<0.05$) with increase in the the quantities of wheat flour substituted. These results contradict past reports (Mbofung et al., 2002) indicating that most composite flours had lower WAC as compared to wheat flours. The quality of maize flour used may explain these differences in WAC. In the present case, the flour fraction used was made of very fine particles (<400 µ) obtained from wet milling. Similar results obtained earlier (Enwere, 1998) confirmed the fact that starch quality in terms of particle size equally plays an important role in the absorption of water.

Dough swelling capacity during fermentation

The maize cultivars used did not affect the swelling capacity of dough from composite flour which was 92 to 94%

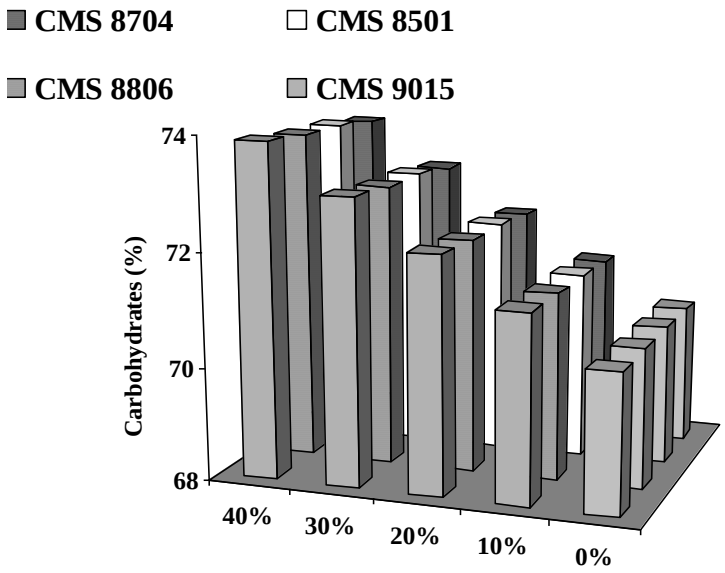

Figure 1. Variation in carbohydrate contents of composite flours as affected by the levels of substitution and maize varieties.

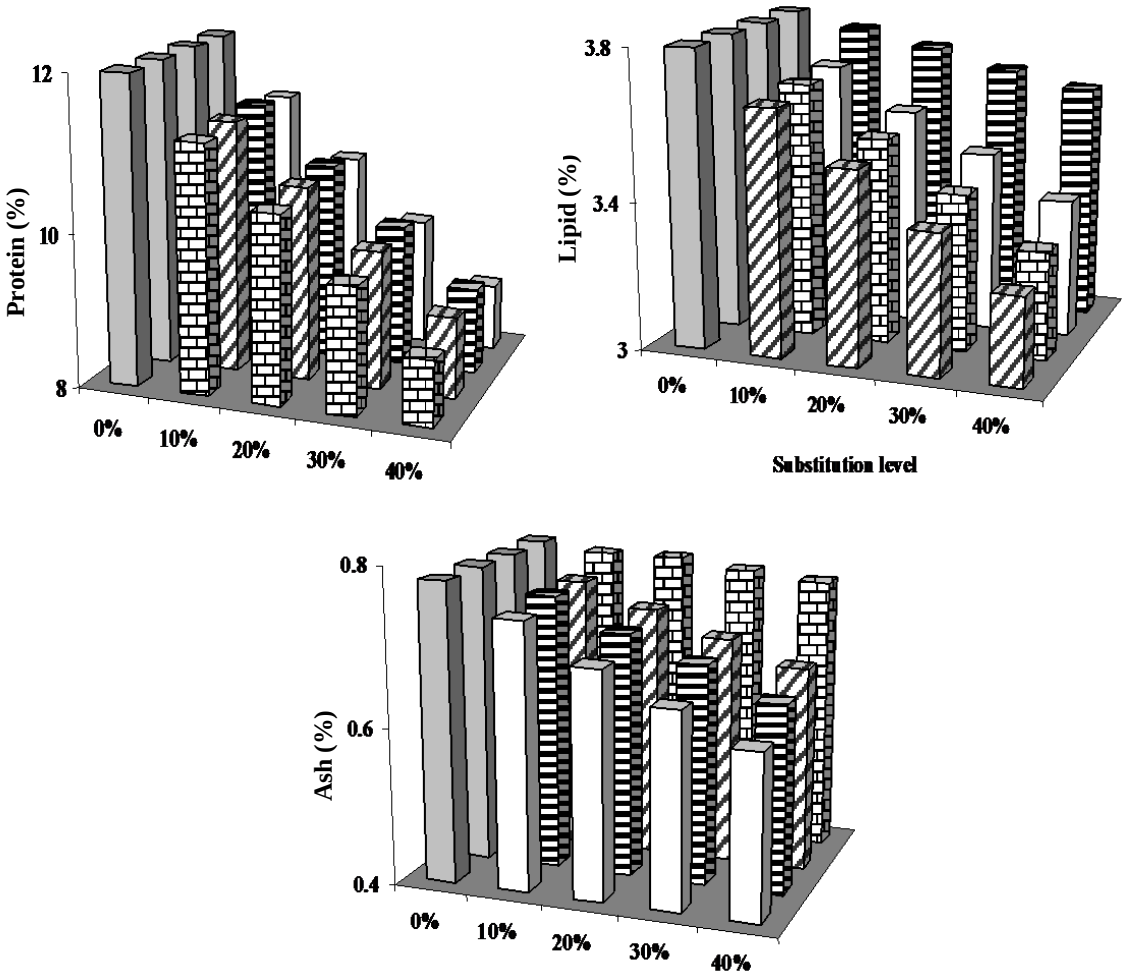

Figure 2. Variation in protein, lipid and ash contents of composite flours as affected by the level of substitution and maize varieties.

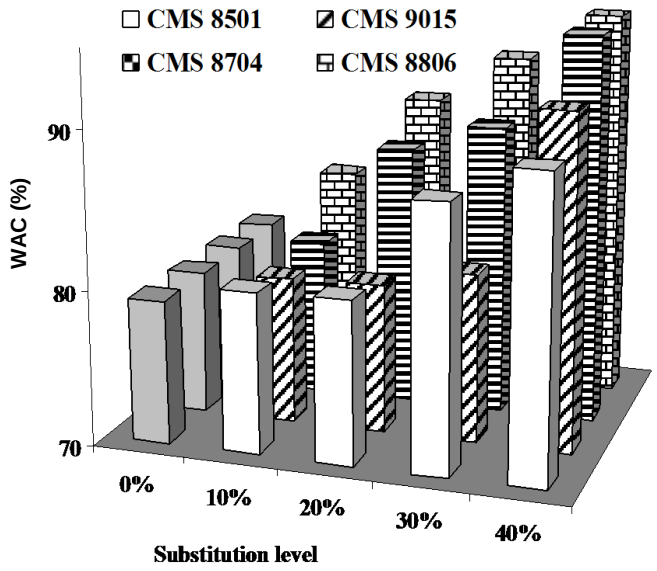

Figure 3. Water absorption capacity of wheat and composite flours as affected by the levels of substitution and maize varieties.

Table 2. Relative rising and weight losses of the dough as function of production parameters.

Dough parameter		Rising of the dough	Weight losses (g)
Substitution rate	0	1.00[a]	0.44[c]
	10	0.94[b]	0.44[c]
	20	0.91[c]	0.46[b]
	30	0.90[c]	0.48[a]
	40	0.88[e]	0.49[a]
Fermentation time in minutes	0	1.00[a]	0.0[e]
	30	0.98[b]	0.17[d]
	60	0.86[e]	0.43[c]
	90	0.88[d]	0.70[b]
	120	0.91[c]	1.02[a]

Means in the same column with different letters are significantly ($p<0.05$) different.

as compared to the control samples from wheat. The incorporation of various levels of maize flour into wheat flour and the fermentation time significantly influenced the rising of fermented dough. Dough swelling capacity (DSC) decreased with decreases in incubation time and increases in maize flour incorporation rates (Table 2). Dough rose to reach a peak after 90 min of fermentation followed by a decline. This was an indicator that dough fermentation time should not exceed 90 min, beyond which cracks appear in the dough ball, and thus facilitate the escape of all the gas trapped, resulting in a drop in its volume. For substitution levels ranging from 10 to 30%, the differences in terms of rising height between the control and the dough from composite flour did not exceed 10%. This was considered as an acceptable margin and therefore 30% substitution level was the possible highest level that would not hamper dough production. The lower gluten content of the composite flours as a result of higher substitution rate could explain the poor rising attributes of these flours. Dough fermentation is known to be affected by flour composition (Pyler, 1979; Delhaye et al., 1984) and this should be taken into account when designing dough products from composite flours. The dillution of the gluten network has affected the volume of gas produced and its retention, resulting in lower dough rising at high substitutions (He and Hoseney, 1991). An inverse trend was obtained in sorghum composite dough with the addition of vital wheat gluten to reinforce the gluten network and gas retention, resulting in higher volume (Cheong and Sun, 1998; Carson et al., 2000).

Table 3. Physical characteristics of fried doughnuts as a function of maize variety and substitution rates of wheat for maize flour.

Dough parameter		Weight of doughnuts prior to frying (g)	Diameters of doughnuts (cm)	Oil absorbed per 100 g (g)	Weight losses after frying (g)
Maize varieties	CMS 8806	39.51[a]	5.11[c]	7.46[e]	10.50[a]
	CMS 9015	39.48[a]	5.10[c]	7.17[f]	9.87[b]
	CMS 8501	39.48[a]	5.01[d]	7.46[e]	10.55[a]
	CMS 8704	39.49[a]	4.96[d]	8.23[c]	9.88[b]
Substitution levels (%)	0	39.31[b]	5.53[a]	6.12[f]	7.88[f]
	10	39.52[a]	5.39[b]	6.54[d]	9.39[d]
	20	39.53[a]	5.17[c]	7.65[d]	10.06[c]
	30	39.54[a]	4.92[d]	8.52[b]	11.28[b]
	40	39.56[a]	4.21[e]	9.07[a]	12.38[e]

Means in the same column with different letters are significantly ($p<0.05$) different.

Table 4. Sensory scores of A*kara* samples containing 0 to 40% maize flour.

Wheat flour substitution level	Origin of flours used for substitution	Sensory attributes of corresponding doughnut (*Makala*)				
		Colour	Texture	Taste	Flavour	Overall acceptance
0% Control	100% wheat	4.41	4.00	4.22	4.11	4.33
10%	CMS 8806	4.00	4.00	4.11	3.96	4.22
	CMS 8501	3.87	4.00	4.00	3.96	4.00
	CMS 8704	3.89	3.88	4.00	4.00	4.11
	CMS 9015	3.89	3.80	4.00	3.96	4.05
20%	CMS 8806	4.00	3.90	4.11	3.96	4.20
	CMS 8501	3.87	3.00	4.11	3.86	4.00
	CMS 8704	3.98	3.86	4.10	4.10	4.00
	CMS 9015	3.89	3.66	4.05	3.94	4.02
30%	CMS 8806	3.98	3.87	4.00	4.02	4.10
	CMS 8501	3.20	2.86	3.66	3.78	3.02
	CMS 8704	3.86	3.85	4.01	4.00	3.96
	CMS 9015	3.90	3,10	3.83	3.61	3.12
40%	CMS 8806	3.64	3.87	3.24	3.10	3.11
	CMS 8501	3.11	2.18	2.46	2.60	2.80
	CMS 8704	3.42	3.58	2.86	2.92	2.98
	CMS 9015	3.06	2.05	2.81	2.45	2.86

Akara physical and sensory attributes

The physical properties of *Akara* are presented in Table 3. Weights of dough nuts were similar irrespective of the maize variety used and levels of substitution. However, weight losses increased significantly in fried doughnut with increases in the fermentation time. Maize varietal effect was expressed on the size and oil retention capacity of finished dough nut. Doughnut from CMS 8704 was significantly higher in fat content, while the amounts of oil absorbed by the dough nut increased significantly with the rate of substitution. Dough nut diameters varied with the maize variety used in the substitution and decreased significantly with increased rate of substitution.

The composite doughnut sensory scores (Table 4) were significantly affected by the level of wheat flour substitution used for its production. Scores for texture and averall acceptance decreased with increases in the amounts of wheat flour substituted. Doughnut from yellow maize (CMS 8806 and CMS 8704) significantly received

higher scores for color and flavor as compared to those from white maize (CMS 8501 and CMS 9015). *Akara* containing 10 and 20% maize flour received scores which were compared with the control independently of maize variety. As the level of maize flour incorporated increased, (30 and 40% level of substitution), the doughnuts had significant respectively ($p<0.05$) lower scores for all the attributes considered. Similar results were previously reported on 20 and 30% sorghum composite bread (Foda et al., 1987; Iwuoha et al., 1997) and 40% sorghum composite bread (Keregero and Mtebe, 1994). Lower scores for flavor and texture *of Akara* made from a blend of wheat and maize (ratio: 60:40) accounted for its least acceptance. *Akara* with best sensory properties was obtained from CMS 8806 and CMS 8704 maize cultivars all of yellow colour.

DISCUSSION

Cameroon improved maize varieties were used to produce composite flour, a major ingredient for *Makala* production. Fine flour from wet milling of maize grains was more suitable to blend with wheat flour as it improved in its functional properties. The influence of maize variety and maize processing method on composite flour was highlighted. Wet milling of maize grain produced fine flour of prarticle size much smaller than those from conventional milling of dry maize. The addition of these small particles of starch in wheat accounted for the improvements on water absorption capacities. With cassava flour, the reduction of the particule size (from 400 to 250 and 180 µm) had a positive effect on bread volume, meaning that particle size index affects the functional properties of composite flours. Although, the various maize cultivars showed specificities in their chemical composition, their wet milling resulted in fine flours of similar composition, thus suggesting the possibility of using any maize cultivars for composite flour production.

The level of wheat flour substituted affected more the functional properties of composite flours as compared to maize variety. Substitution of wheat for maize flour reduced the protein contents and particularly the gluten contents well known to affect gas production and consequently swelling of the dough. Previous results have shown that the major factors responsible for the bulk of water uptake were proteins contents, quality of starch and to a lesser extent, cellulose contents (Sefa-Dedeh et al., 2001; Martin and Fitzgerald, 2002). Substituting wheat for maize affected these factors resulting in doughnut of different sensory features. In sorghum, it was observed (Fliedel et al., 1989; Njintang et al., 2001) that flour of finer particle sizes would affect the particle size index giving a larger surface area in contact with water and thus increasing WAC which plays major roles in the food preparation processes. The range of application of most flours as food ingredients is dependent to a large extent on their ability to hydrate and thus imparting their desired functional property on the food system.

The sensory analysis done was useful is assessing the effect of maize variety and substitution level in the composite doughnut. It provided a basis for determining acceptance of composite dough nut by potential consumers. Sensory evaluation showed that all the various dough nuts produced were of acceptable quality as none was graded unfit for consumption and suggesting that blends of wheat and maize (ratio between 90:10 and 60:40) can serve for *Akara* production. Wet milling selected for maize flour production is a routine practice carried out daily by village women and can be achieved through the machinery locally available. This low cost of production of one ingredient will reduce the overall cost of production of doughnut and ensure more revenue to village women who are the main producers of doughnuts. The use of composite flour is of great importance to small scale producers with very little capital for rural women. Mixing is a simple process that can easily be adopted by women producing doughnuts. Comment from panelists showed that composite dough nuts were slow digesting and therefore could sustain farmers much longer on the field for long working hours. This argument was strongly in favor of the production of doughnuts from composite flour.

Conclusion

The maize cultivars developed were found suitable for the production of fine fraction in wet milling for composite flour. The differences in the cultivars did not affect the functional properties of composite flours, thus suggesting their possible use for large scale production. The acceptability of *Akara* decreased with increasing substitution rates from 10 to 40%. These results suggest that *Akara from* composite flour containing up to 40% maize can satisfy the consumers' demand in Sub-Saharan areas, especially in North Cameroon.

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Full Length Research Paper

The effect of *Withania coagulans* as a coagulant on the quality and sensorial properties of Tofu

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Tofu is a nutritional, gel-like soy food. The present study was carried out to investigate the effects of *Withania coagulans* extract on the soymilk coagulation for producing tofu. For this purpose, soymilk was coagulated by *W. coagulans* extract, and the properties of the prepared tofu were analyzed. The results indicate that the extract of *W. coagulans* as a coagulant significantly ($p<0.05$) reduced yield and moisture content when compared with calcium sulfate tofu, but fat and protein contents of *W. coagulans* tofu and calcium sulfate tofu were the same. Results of sensory evaluation show that the sensorial properties of *W. coagulans* tofu are not different from that of calcium sulfate tofu. In this way, it was concluded that the extract of *W. coagulans* fruits could be used as a natural coagulant in coagulation processes of soymilk.

Key words: *Withania coagulans* soymilk, tofu, coagulant, syneresis.

INTRODUCTION

Soybean is a major source of protein, having a protein content of 35-40% on a dry basis, making it a relatively inexpensive source of protein for human consumption (Derbyshire et al., 1976). Soybeans are processed into different kinds of products such as tofu one of the most important and valued soy foods throughout the world (Mujoo et al., 2003), especially in East and South Eastern Asian countries due to their inexpensive and high quality protein (Birthal et al., 2010). Use of tofu and soy-products in the Western countries are in an increasing rate (Atkinson et al., 2002).

Tofu preparation generally includes soaking and grinding of soybeans in water, filtering, boiling and coagulation of soymilk. The yield, quality and texture of tofu are influenced by many factors such as variety of soybeans (Shen et al., 2006), processing methods (Kyoko, 1979; Shih et al., 2006) and type and concentration of coagulants (Lim et al., 2006; Shen et al., 2006; Sun and Breene, 1991).

Usually, calcium sulfate and glucono -δ-lactone are used more than other coagulants on an industrial scale for the tofu making (Lim et al., 2006; Sun and Breene, 1991; Tsai et al., 2006; Wang and Hesseltine, 1982). Tofu is generally known as a salt or acid coagulated soy protein gel, with soya lipids and proteins and other constituents trapped in its networks (Kohyama et al., 1995).

Most studies about tofu are available in the literature and used chemical coagulants for preparation of tofu (Beddows and Wong, 1987; DeMan et al., 1987; Schaefer and Love, 2007; Shen et al., 2006; Sun and Breene, 1991; Wang and Hesseltine, 1982). The extract of *Withania coagulans* is considered as a natural coagulant (Dastur et al., 1949).

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W. coagulans Dunal (Family: Solanaceae) is in the form of a shrub and it is usually recognized as Indian cheese maker. *W. coagulans* grows in Pakistan, Afghanistan and India as well as in southern regions of Iran. This vegetative coagulant has been used for preparation of traditional cheeses from raw cow's milk (Dastur, 1949; Roseiro et al., 2003). The main components of the berries other than the milk-coagulating enzyme are esterases, free amino acids, fatty oils (an essential oil), alkaloids and withanolides (Atta-ur-Rahman et al., 2003).

The aim of this study was to examine the effects of Vegetative coagulant from *W. coagulans* on the quality and yield of tofu making, in an attempt to use this enzyme as a new local source of coagulant in preparation of tofu.

MATERIALS AND METHODS

Soybean used for tofu making was purchased from a local market (city of Mamaghan, Iran). Calcium sulfate and other chemicals were purchased from Merck Company (Darmstadt, Germany). All chemical reagents were in analytical grade.

Dried fruits of *W. coagulans* were collected from wild plants growing in the Southeast of Iran (Sistan and Baluchestan Province).

Enzyme extraction

According to Naz et al. (2009), dried fruits of *W. coagulans* powdered by grinder (Bel-Art Products, Pequannock, NJ, USA) and enzyme extract was obtained by soaking the powder in 0.85% saline solution at 4°C for 24 h. The extract was filtered to obtain crude extract. This coagulant was kept in 4°C for future uses.

Preparation of soymilk

Soymilk was prepared according to the method of Noh et al. (2005) with a few modifications. Soybeans were soaked in water at room temperature for a period of 10 h, rinsed and drained, then ground with water at a bean: water ratio of 1:4 by using a waring blender (Bel-Art Products, Pequannock, NJ, USA) for 5 min at high speed. After grinding, the resultant slurry was cooked under stirring condition and held at 95°C for 15 min. The slurry was squeezed handy with a muslin cloth and pressed to remove the soy residue (okara), to obtain a soymilk of 12–14° Brix. A single batch of soymilk was made from the soybean and was used for the preparation of tofu by using each coagulant.

Preparation of tofu

According to Liu et al. (2004), 100 ml portion of prepared soymilk (75°C) was poured in a 250 ml beaker, and food grade 0.5% calcium sulfate solution was added to the solution with constant stirring. Stirring was stopped after complete coagulation (10 min) and content was kept without stirring for 15 min at room temperature. Tofu gel was formed and cooled to 20°C without moving from the beaker, and then kept at 4°C until the next day for analysis and yield rate measurement.

For preparation of *W. coagulans* tofu, different concentrations of prepared *W. coagulans* extract (0.25, 0.5, 1 and 1.5%) were added in a 100 ml of soymilk (37°C) with a little stirring. The content of beaker was kept for 10, 20, 30 and 40 min at 35, 37, 40 and 45°C and the same procedure was used for calcium sulfate tofu.

Analysis

Yield, moisture, protein, pH and fat analyses

Before analysis, when the tofu reached room temperature, tofu carefully was removed out from the beaker and at the same time, whey was removed and fresh tofu-gel was weighted.

Yield of tofu was calculated as fresh weight of tofu-gel obtained from a specified amount of the soybean used for its making.

Moisture content was determined by drying 5 g of fresh tofu at 105°C in an oven to constant weight (Tsai et al., 2006). Total protein was determined by the micro Kjeldahl method (AOAC, 1995). The factor N×6.25 was used to convert nitrogen to protein. The fat content of tofu was determined by the Soxhlet method (AOAC, 1995). pH of the tofu samples was measured with a digital pH meter with a glass electrode (Metrohm AG, Switzerland).

Syneresis rate

After equilibrating in the room temperature (20°C) for 1 h, the tofu-gel was carefully removed wholly from the beaker and cut cross-wise with a sharp knife to pieces with diameter of 1.5 cm.

Syneresis was evaluated by employing the modified method of Armstrong et al. (1994). After cutting, six pieces of tofu-gel samples were put on 20 mesh stainless steel grid in a plastic box. The grid was supported by four sticks. The exuded liquid was allowed to be separated from tofu-gel. The box was covered with plastic to prevent evaporating. Since the liquid was released slowly, six pieces of tofu-gel samples were stored in the box for 10 h in refrigerator. The liquid quantity exuded during the 10 h was used to calculate syneresis rate:

$$\text{Syneresis}(\%) = \frac{\text{Weight of water exuded from the sample in 10h}}{\text{Weight of sample}} \times 100$$

Sensory evaluation

Thirty-two (32) untrained panelists, composed of adult males and females, scored the sensory characteristics of tofu-gel. Tests on overall acceptability, color, flavor and texture were conducted using a 5-point hedonic scale (5 = excellent, 1 = not good) for each attribute. All samples were coded and presented in a randomized arrangement.

Statistical design and analysis

Experiments were based on a randomized complete block design. All extraction experiments and analyzes were performed seven times and values are reported as means ± SD as obtained. Analysis of variance was conducted for each data collected, using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Duncan's multiple range tests was used to determine differences between the two prepared tofus. The Statistical tests were conducted at the 5% probability level.

RESULTS AND DISCUSSION

W. coagulans tofu coagulated only when 1% prepared *W. coagulans* extract was added in a 100 ml of soymilk

Table 1. Syneresis, yield and contents of moisture, protein, fat, pH of prepared tofu with two different coagulants.

Sample	Yield					
	Soybean (kg/kg)	Moisture (%)	Protein (%, d.b.)	Fat (%, d.b.)	pH	Syneresis (%)
Calcium sulfate tofu	3.13[a]	86.75±0.16[a]	52.70±1.41[a]	28.73±0.63[a]	5.44±0.25	15.4±0.3[a]
Withania coagulans tofu	2.19[b]	85.98±0.33[b]	52.25±1.53[a]	28.44±0.81[a]	5.08±0.12	17.0±0.5[b]

Values are means ± standard deviation. Means with the same letter within columns are not significantly different from each other (P < 0.05).

Table 2. Effect of *Withania coagulans* on sensory characteristics of tofu.

Sample	Flavor	Color	Texture	Overall acceptability
Calcium sulfate tofu	2.15[a]	2.9[a]	2.84[a]	2.28[a]
*Withaniacoagulans*tofu	2.53[a]	2.87[a]	2.81[a]	2.44[a]

Means with the same letter within columns are not significantly different from each other (P < 0.05).

(37°C) with a little stirring and then it was kept for 20 min at 37°C. These results are in the line with the results of Naz et al. (2009). The main objective of their study was to purify the protease from *W. coagulans* and partially characterize it, and they concluded that 37°C and 15 μLmL^{-1} of enzyme would be the best concentration for coagulation.

Yield and composition of tofu

The composition and yield of tofu samples are shown in Table 1.

No significant differences were found between protein and lipid content of tofu coagulated by these two coagulants. The formation of tofu gels entangles proteins, fats, hydrocarbonates, and air within the gel networks. The aggregated soy protein networks which are full of air bubbles, scaffold the tofu gel. The networks form numerous boundaries among the media (Kohyama et al., 1995; Saowapark et al., 2008).The yield of *W. coagulans* tofu was lower than that of calcium sulfate tofu. The lower yield of *W. coagulans* tofu was reflected by the lower moisture content because tofu yield and moisture contents are highly correlated (Cai et al., 1997). These results are in the line with the results of Noh et al. (2005). They studied quality of tofu as affected by freezing treatment of soybeans and reported that the lower yield and moisture content of tofu from frozen soybeans may be ascribed to the denser and more compact structure, which made water easily release from the curd during pressing. The variation in the moisture content of tofu prepared with different coagulants is probably due to the differences in the gel network affected by the ionic strengths of the coagulants and/or the effect of the different anions on the water-holding capacity of the soy-protein gels (Wang and Hesseltine, 1982).The pH of *W. coagulans* tofu was lower than that of calcium sulfate tofu; these results are in line with the results of Pezeshki et al. (2011) which reported that pH was significantly (p< 0.05) lower in cheeses made with *W. coagulans*as coagulant than pH of cheeses with animal or fungi rennets. A decrease in pH was described as essential for the coagulation of soy proteins by many previous researchers (Beddows and Wong, 1987; Lu et al., 2006). The lower pH of *W. coagulans* tofu may reflect the isoelectric precipitation of soy-proteins by the release of protons from δ-lactone (Smith, 1978) because Withanolides are a group of ergostanolides, generally having a δ-lactone in the side chain (Abraham et al., 1968).

Syneresis rate

It is recognized that *W. coagulans* significantly (p<0.05) increased the tofu's syneresis rate. Probably due to an unspecific proteolitic activity of *W. coagulans* enzymes (Pezeshki et al., 2011), however, increase of syneresis from the curd could be due to increase in bonding occurring during storage, making the protein matrix more dense and compacted (Sun and Breene, 1991) and also differences between microstructures may be responsible for different physical properties between the two kinds of tofu gels (Liu et al., 2004).

Sensory evaluation

Table 2 shows the results of sensory evaluation of tofu for color, flavor, texture and overall acceptance. The results were expressed on a 5-point hedonic scale.

There were no significant differences between sensory characteristics of tofu coagulated by these two coagulants. This means that extract of *W. coagulans* had few effect on the sensory attributes of tofu.

Conclusion

This study has confirmed the possibility of replacing calcium sulfate by extract of *W. coagulans* for the coagulation of soybean milk in the preparation of tofu. Furthermore, the study has demonstrated that *W. coagulans* tofu had fat and protein content similar to calcium sulfate tofu but both yield and moisture content were lower. In addition, using *W. coagulans* could reduce heating process needed for coagulation of soymilk, without impairing the quality of tofu, and the cost of tofu preparation is reduced because of the cheaper price of *W. coagulans* than that of calcium sulfate

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Full Length Research Paper

Thermal oxidative alteration of sunflower oil

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Sunflower oil is extensively used in frying in Algeria as an alternative to olive oil due to its low cost. However, the high level of unsaturated fatty acids (FA) contained in sunflower oil enhances its susceptibility to oxidation. In our study, the sunflower oil was heated at 99±2°C with incorporation of 9 L of oxygen/second for 52 h continuously in the absence of foodstuff. Heating polyunsaturated fatty acids (PUFAs) in the presence of air causes a greater degree of lipid peroxidation. The oil oxidation degree was monitored through several physicochemical analyses. The products of thermal oxidation were monitored using UV- spectrophotometric method and Fourier transform infrared spectroscopy (FT-IR). Compared to fresh oil, the free-fatty acid contents, peroxide value, density and moisture of the thermally oxidized sunflower oil increased. In addition, the iodine and saponification values decreased during thermal treatment. The treatment applied had a negative effect on FA composition; the most significant effects were on C18:2, C18:1 and C16:0 contents. Analysis of chromatographic profile of thermoxidized sunflower oil showed a reduction in linoleic acid (LA) and an increase in oleic and palmitic acids; decrease of linoleic acid content is used as an indicator of lipid oxidation. Moreover, during the early stages, conjugated dienes (CDs), absorbing at 233 nm, were formed upon decomposition of hydroperoxides. In our study, the early stages of lipid oxidation were measured by UV-spectrophotometric method. Hydroperoxides broke down into secondary products and were revealed by FT-IR; these scission products are generally odoriferous by nature. The C=O stretching band at 1739-1724 cm^{-1} of the aldehydes was much more intense. Formation of conjugated double bond systems and the isomerisation of *cis* to *trans* double bonds was observed in the C=C stretching region at 980 to 965 cm^{-1}. The results obtained reveal that even fresh oil contains products of peroxidation and isomerization of C18:2, n-6; indeed, CDs can be produced during the refining process of oil. The treatment applied increased the rate of these products and conferred a marked rancid taste and a thick texture to the thermoxidized sunflower oil.

Key words: Sunflower oil, Linoleic acid, thermally oxidative treatment, alteration level.

INTRODUCTION

Sunflower oil is a high-quality edible oil. It is used in cooking, frying, and in the manufacture of margarine and shortening and considered by some as desirable as olive oil. Sunflower oil was selected in this study due to its high use in food as it is a rich source of linoleic acid. Furthermore, it is light in taste and appearance and has a high vitamin E content compared to other vegetable oils (Shahidi et al. 1992).

At high temperature and in the presence of air, many chemical reactions can be observed in oil: hydrolysis, polymerization, oxidation and isomerization (Rossell, 2001). Thus, new and unstable compounds potentially

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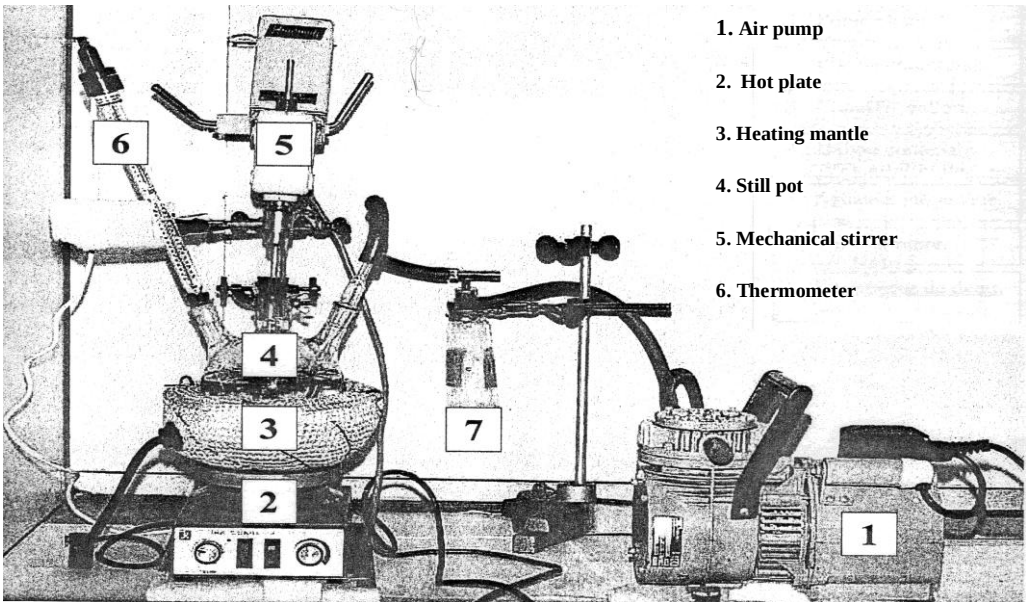

Figure 1. Mounting of thermal oxidation device.

toxic at low concentrations may be generated after destruction of the linoleic acid (Nawar, 1996; Min and Boff, 2001).

The objective of this study was to evaluate the deterioration levels of sunflower oil subjected to thermoxidative treatment (in the absence of foodstuff) according to the laboratory instrumental method developed by Drozdowski and Szukalska (1987) modified by Blanc-Gondardmary et al. (1989). There is no standard method to detect oxidetive changes during the entire process, and a combination of different analytical techniques is usually required (Gray, 1978; Frankel, 1993; Warner and Eskin, 1995). In our study, the primary oxidative products were monitored through peroxide value (PV), loss of unsaturated fatty acids, conjugated diene value, and others. Secondary changes are measured by Fourier transform infrared (FT-IR) spectroscopy, this latter gives information about the different functional groups present in the sample; thus, it is not limited to just one kind of compound like the indices mentioned above (Muik et al., 2005).

MATERIALS AND METHODS

Sunflower oil was purchased at a local market in Tizi-Ouzou, Algeria. This oil was obtained from the seeds of *Héliantalus annus linnaeus* containing 40% of oil. Sunflower oil-like most vegetable oils is composed mainly of triacylglycerols (98 to 99%), and a small fraction of phospholipids, tocopherols, sterols, and waxes (all of the latter are commonly referred to as the ‘‘unsaponifiable fraction’’). Sunflower oil is characterized by a high concentration of linoleic acid, followed by oleic acid and it has a low content of palmitic acid compared with other oils (Grompone, 2005). Decrease of linoleic acid content is used as an indicator of lipid oxidation. The mechanism of lipid oxidation changes significantly at elevated temperatures and depends strongly on oxygen availability. In our study, the sunflower oil (700 ml) was heated at 99±2°C with incorporation of 9 L of oxygen / s for 52 h continuously in dark. Figure 1 show the apparatus setup used in thermally oxidative treatment.

After treatment, thermoxidized sunflower oil was kept under nitrogen before sealing airtight in a glass bottle and stored at -20°C until further analysis. The degree of oxidation of the oil was monitored through several physicochemical analyses. The following parameters were studied: free-fatty acid (FFA) contents, peroxide value (PV), refractive index (RI), humidity (H%), iodine value (IV), saponification value (SV) and density (AOCS, 1989); fatty acid (FA) composition (AOAC, 1999); specific extinction at 232 nm (*K*232) and 270 nm (*K*270) related to the content of conjugated dienes (CDs) and trienes (CTs) of linoleic acid (18:2 n-6) respectively was determined using UV spectrophotometer (IUPAC, 1987); secondary lipid oxidation products were determined using FT-IR spectroscopy.

Statistical data analysis

The average comparison was realized by the analysis of variance (ANOVA) with Stat. Box. Edition 6.4. The significance level was selected at $p < 0.05$.

RESULTS

The study shows that compared to fresh oil, the FFA content, PV, RI, density and moisture of the oxidized sunflower oil increased from 0.093 to 1.25%, 5.83 to 152.5 meq/kg, 1.461 to 1.476, 0.910 to 0.985 and 0.100 to 2.006 respectively. The IV and SV decreased from 125.84 to 80.51 g I_2/100 g and 192.60 to 183.79 mg KOH/g respectively. In addition, a reduction in linoleic acid (from 58.14 to 40.59%) and an increase in oleic acid

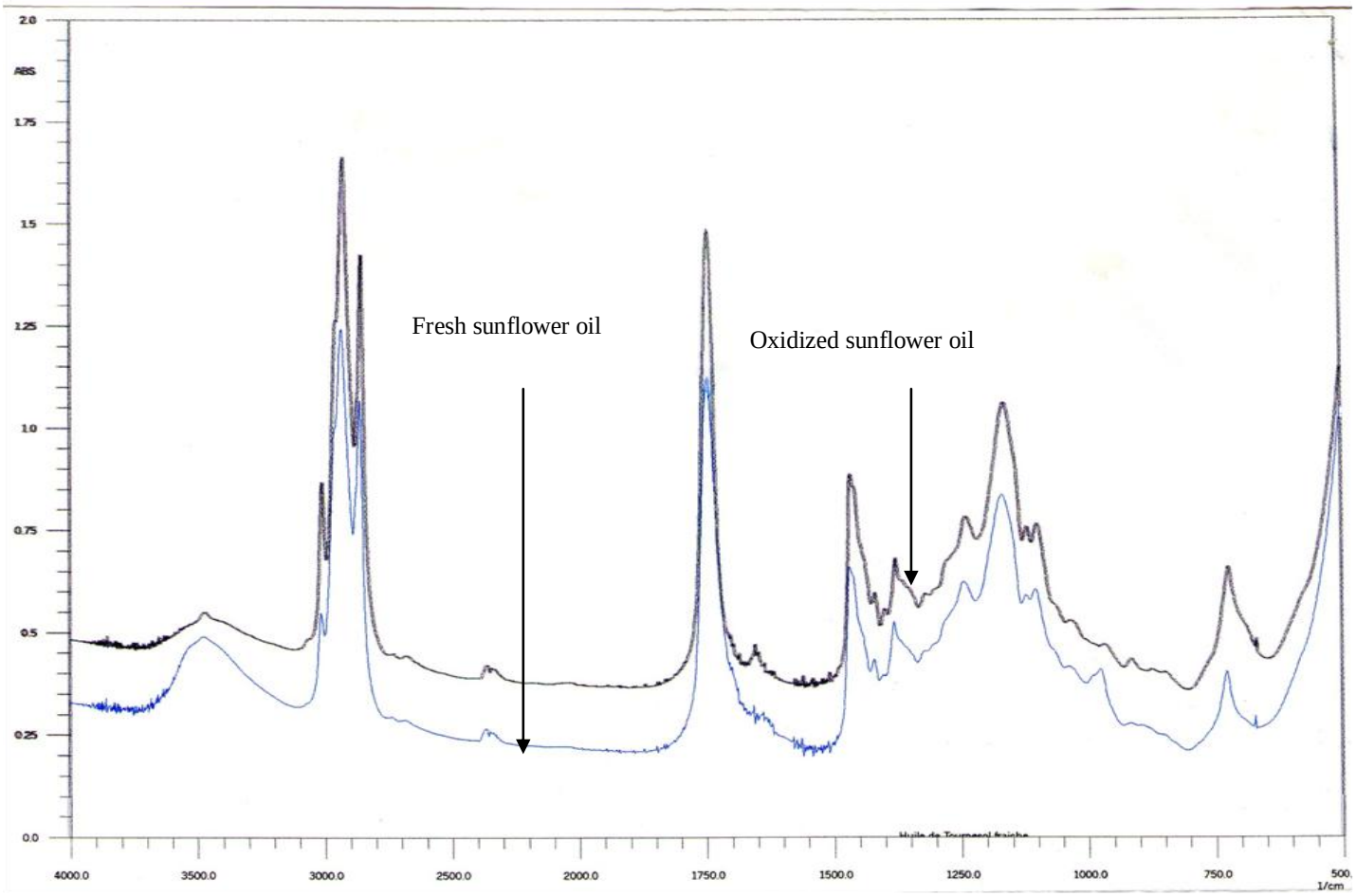

Figure 2. FT-IR spectra of fresh and thermoxidized sunflower oils (4000 - 500 cm^{-1}).

(from 33.500 to 46.04%) in relation to fresh sunflower oil were observed. Decrease of linoleic acid content was used as an indicator of lipid oxidation. Conjugated double bonds (C=C–C=C) values (expressed as extinction values at 232 nm), which are primary products, increased from 0.0002 to 0.033, whereas conjugated trienes values at 232 nm ranged from 0.423 to 0.655. Formation of aldehydes, ketones and other secondary oxidation products, revealed by FT-IR spectroscopy, in thermoxidized oil was accelerated by constantly bubbling air into the oil during heating (Figure 2). Then, flavour, aroma and taste of the oil were affected. Also, the formation of conjugated double bond systems and the isomerisation of *cis* to *trans* double bonds as observed in the C=C stretching region contributed to change of the density and viscosity of the thermoxidized sunflower oil.

Nevertheless, part of the double bonds of the linoleic acid remained at configuration *cis* (C=C–C–C=C); this essential FA was proportioned at 40.59% in oxidized oil. Our results show a strong increase of the band located at 1667 to 1639 cm^{-1} which can be assigned to *cis* double bonds in oxidized oil compared to fresh oil.

DISCUSSION

In order to document the thermally oxidative treatment influence on sunflower oil, an exhaustive examination was needed for both physical and chemical criteria of this oil (Table 1). The results of various investigated parameters led to the conclusion that the treatment applied in our study caused a high level of deterioration of this oil.

Compared to fresh oil, the thermoxidized oil contained approximately 13 times more total FFA than fresh oil (0.093 *vs*. 1.25%) and its acidity content was higher (P=0) than fresh oil (0.182 *vs*. 2.51 mg KOH/g). Formation of FFA might be an important measure of rancidity of foods. FFAs are formed due to hydrolysis of triglycerides and may get promoted by reaction of oil with moisture (Freja et al., 1999). The advanced deterioration of thermoxidized oil was due to its strong humidification. The treatment caused a very significant (P=0) rise in humidity (2% *vs.* 0.1%). Humidification of oxidized oil would be due to the formation of water and volatile compounds which constitute the final products of decomposition of hydroperoxides.

Table 1. Physico-chemical properties of fresh and thermoxidized sunflower oil.

Characteristic	Fresh oil	Thermoxidized oil
Physical state at room temperature	Fluid	Thick texture, flavor faded
Peroxide value (meq/Kg)	5.83 ± 0.76	152.5 ± 5.33
Acid value (mg KOH/g)	0.182 ± 0.04	2.51 ± 0.11
Free Fatty Acids (%)	0.093 ± 0.02	1.25 ± 0.07
Iodine value (g I_2/100g)	125.84 ± 1.59	80.51 ± 1.37
Saponification value (mg KOH/g)	192.60 ± 2.91	183.79 ± 1.32
Refraction index	1.461 ± 0.037	1.476 ± 0.023
Density at 20°C (g/ml)	0.910 ± 0.018	0.985 ± 0.010
Moisture (%)	0.100 ± 0.012	2.006 ± 0.221
C16 :0 content (%)	6.14 ± 0.451	9.48 ± 0.367
C18 :1, n-9 content (%)	33.500 ± 2.317	46.040 ± 3.939
C18 :2, n-6 content (%)	58.140 ± 2.475	40.590 ± 0.829

Values are mean±standard deviation of triplicate determinations.

Table 2. CDs and CTs extinctions values in both oils.

λ (nm)	Extinction values « K »	
	Fresh oil	Thermoxidized oil
200	-	0.112
232	-	0.036
250	0.0002	0.033
262	-	0.027
268	0.293	0.407
270	0.423	0.655

In addition, this treatment decreased the global unsaturation of thermoxidized oil. The IV is a measure of the total number of double bonds. During the oxidation process, a very significant decrease (P=0) in IV was observed (125.84 *vs.* 80.51). This reduction was more pronounced than those obtained by Blanc-Gondardmary et al. (1989). This variation was due to the more prolonged time of heating applied. Decrease in IV is an indicator of lipid oxidation (Naz et al., 2004). Our results obtained by gas chromatography (GC) showed a dramatically reduced level of unsaturated FA.

PV is a widely used measure of primary lipid oxidation indicating the amount of peroxides formed in fats and oils during oxidation (Ozkan et al., 2007). Our result show a considerable (P=0) increase (5.83 *vs.* 152.5 meq/Kg) in PV after subjecting the oil to oxidation conditions. Such increase in PV had been reported by Neff et al. (1994), Liu and White (1992).

Oxidation of polyunsaturated fatty acids (PUFA) leads to primary and secondary oxidation products. Compounds and amounts of these products vary, depending on the oxidative conditions. To evaluate the oxidation state of the oil, the following parameters have been measured in the two oils. The UV spectrophotometric absorption at 232 and 270 nm, expressed as *K*232 and *K*270, measures the formation of CD and CT, respectively. The *K*232 value increased strongly in oxidized oil reflecting that much CD was formed (Table 2). The spectrophotometric absorption at 270 nm showed for the thermoxidized oils a tendency that resembles very well that of the band located at 833 to 866 cm^{-1} for hydroperoxides, 1739 to 1724 cm^{-1} for aldehydes and 1724 to 1709 cm^{-1} for ketones.

In addition, our experimental conditions supported the peroxidation of linoleic acid and increased the polarity of oil revealed by measurement of PV and IR spectrophotometry at 3636 to 3571cm^{-1} respectively. In the frame of this study, we compared the FA composition of thermoxidized and fresh sunflower oil.

Thermal treatment led to dramatical differences in sunflower oil FA composition; the linoleic acid decreased upon heating, which explained the increase in oleic and palmitic acids. This has been previously reported in heated sunflower oil (Juaneda et al., 2003). The sensitivity of the double bounds of linoleic acid to the combined action of heat and oxygen would be responsible for this finding. Crapiste et al. (1999) demonstrated that as alteration advanced, there was a continuous decrease of

unsaturated fatty acids, particularly linoleic acid, being more pronounced at the highest temperature. It resulted in an increase in the oleic acid to linoleic acid ratio (o/l), indicating a preferential use of linoleic acid in oxidation reactions. Otherwise, Marmesat et al. (2009) observed, in high linoleic sunflower oil, that the oxidation took place mainly in the linoleyl group of the triacylglycerols, while the loss of oleyl group was minimum throughout the total oxidation period. However, in high oleic sunflower oil, due to the high relative content in oleic acid, the decrease of oleic acid was similar to that found for linoleic acid.

Different FA isomers were formed during heating and higher differences were found between their contents in the thermoxidized and fresh sunflower oils. The hydroperoxides and CD, relatively stable, absorbed at 232 nm. They are quantifiable by UV spectrophotometric method (Laguerre et al., 2007). These primary products of oxidetion were formed highly in oxidized oil; an extinction of 0.073 was noted. Hydroperoxides can be decomposed into secondary products. Aldehydes and ketones have been reported as major secondary oxidation products. For oxidized oil, the value of this parameter was much higher than for the fresh oil (1.122 *vs.* 0.716). Accumulation of these secondary products in oxidized oil caused deterioration of its flavor.

FT-IR spectroscopy has been used to investigate the chemical changes taking place during lipid oxidation in several edible oils. Differences among the spectra of the fresh and oxidized oils were located at all of the bands suggested by Mazliak (1968) (Figure 2). The intensities of these bands were higher in oxidized oil than in fresh oil which reflects the high level deterioration of oil. Thermoxidized oil contained more hydroperoxides than fresh oil; part of this primary product was converted into secondary products; the C=O stretching band of the aldehydes was much more intense. Thermal oxidation led to strong increases of the bands at 833 to 866 and at 1739 to 1724 cm^{-1} respectively.

Oxidative degradation of sunflower oil was accelerated by heating at 100°C combined to oxygen insufflated. The formation of conjugated double bond systems and the isomerisation of *cis* to *trans* double bonds was observed in the C=C stretching region (980 to 965 cm^{-1}). Thermal oxidation led to strong increases of the band at 980 to 965 cm^{-1} assigned to CD. Thick texture of thermoxidized oil can be attributed to high amounts of CD and CT produced, which is also reflected in *K*270 and *K*232 of this oil; this principal parameters reflect the isomerisation of *cis* double bonds to *trans* double bonds reflecting change of the density and texture of this oil.

In addition to ketones and aldehydes, oxidized oil exhibited a very strong band at 1761 cm^{-1} which corresponds to the C=O stretching (1761 cm^{-1}) of the formic acid (HCOOH). In fact, this compound contributed to the deterioration of the organoleptic quality of oil. This acid will be converted into CO_2 and H_2O, other final products of oxidation.

Conclusion

From the results of various investigated parameters, it can be concluded that the treatment applied to the sunflower oil, which is heating at 100°C with continuous air insufflations for 52 h, caused a high level deterioration with development of oxidative rancidity. Results obtained spectrophotometrically showed very strong band at 980 to 965 cm^{-1} which corresponds to the C=C stretching vibration in conjugated systems; the C=O stretching band of the aldehydes (1739 to 1724 cm^{-1}) was much more intense in oxidized oil regardless of treatment. Over-consumption of these components can be detrimental to health. Significant losses in the essential fatty acid (linoleic acid) were also evident in sunflower oil subjected to treatment.

In further work, we plan to apply this technique to the study of related oxidation processes in biological systems that are gaining increased attention with regard to possible connection between lipid oxidation and pathological events.

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Full Length Research Paper

Cyanide and selected nutrients content of different preparations of leaves from three cassava species

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Cassava leaves are largely consumed as vegetable in African, but contain a toxic compound, cyanide. To ascertain their safety and contribution to human nutrition, after a number of pre-treatments preceding their boiling in water, cyanide, vitamin C, β-carotene, crude protein, iron, calcium, phosphorus, potassium and zinc contents were assessed in leaves from bitter, sweet and wild cassava species, boiled for 15 and 30 min after differently processed by: (1) pounding un-dried (UND), (2) drying before pounding (DBP) and (3) drying after pounding (DAP). Blanching headed drying was done in a tunnel solar dryer. Results showed that cassava species, processing procedures, and boiling time significantly ($p < 0.05$) reduced cyanide and the nutrients. However, except vitamin C, eliminated to almost nil, other nutrients were retained at considerable levels. Sensibly decreased by drying and/or boiling, cyanide levels ranged from 32 - 50 mg HCN/kg (dry matter basis) after boiling for 30 min. These levels, above the recommended level (10 mg HCN/kg) for foods, were safe with regard to cyanide toxicity based on the fact that the vegetable is served in small quantities as side food. consumed quantities of relishes as side foods. Nevertheless, it was advisable not to make them the everyday foods, especially to lower body weight such as children, and to extend time of cooking.

Key words: Cassava leaves, cassava species, processing procedures, cyanide and nutrients, Rwanda

INTRODUCTION

Cassava is a very important crop in the tropics and a staple food for over 800 million people (Nassar et al., 2007), growing over a range of climates and altitudes and on a wide variety of soils (FAO/IFAD, 2005). In Africa, Rwanda included, cassava is primarily used as food, consuming roots as starchy food (Nweke et al., 2002), but leaves are also largely consumed as green vegetables (Achidi et al., 2005).

Producing a valuable and safe food from cassava and cassava leaves involve certain challenges. In fact, cassava and cassava leaves contain cyanide, in the form of cyanogenic glucosides, primarily linamarin and small lotaustralin (Uyoh et al., 2007). The cyanogenic glucosides are distributed throughout the cassava plant, with highest levels in leaves (Etonihu et al., 2011). Under high temperature, pressure, and use of enzyme (linamarase), or mineral acids, cyanogenic glucosides are decomposed into acetone cyanohydrins which, at pH above 5 or temperatures above 35°C, is broken down, spontaneously, into poisonous compound, hydrogen cyanide (HCN) (Siritunga and Sayre, 2004). Cyanohydrins are the most dangerous form of the cyanide because at the elevated pHs and temperatures present in the human body, it rapidly decomposes to release the poisonous

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hydrogen cyanide. The continued consumption of high dietary cyanogens has been linked with a number of chronic health disorders, and occasionally death, depending on the level of cyanogens, frequency of cyanogens exposure and, quality and quantity of protein intake status (Cliff et al., 2011; Nhassico et al., 2008; CCDN, 2007). For the human body detoxification, unbound cyanide is converted to less toxic thiocyanate (SCN) and is excreted in the urine. The synthesis of thiocyanate requires sulphur-containing amino acids, as a consequence of protein intake (CCDN, 2007).

Despite the presence of the poisonous component, numerous publications have provided evidence on potential contribution of cassava leaves in human nutrition by providing protein, minerals and vitamins, depending on preparation techniques (Akinwale et al., 2010; Mulokozi et al., 2007; Ayodeji, 2005). Faber and Van Jaarsveld (2007) revealed that improved handling, such as optimizing time of thermal treatment, drying process and preliminary preparations can preserve quality of treated food. In Rwanda, leaves from three species of cassava, bitter (*Manihot utilissima),* sweet (*Manihot dulcis)* and wild (*Manihot glaziovii)* are valued and highly utilized as green vegetables. They are usually cooked freshly harvested, but preservation by sun-drying to extend their shelf life is also reported by Umuhozariho et al., (2011). The direct exposure to sunlight is known to reduce the quality (colour and vitamin contents) of the final product (MMA, 2008). However, solar drying is reported among strategies to combating nutrients losses in processed food stuffs and extending the availability of the nutrient-rich foods, beyond the season in which they are in abundance (Oguche Gladys, 2011; Thompson and Amoroso, 2011).

The present study was undertaken to improve drying of cassava leaves by using tunnel solar dryer and evaluate levels of cyanide and nutrients in the dried and un-dried leaves, from the three cassava species and after preparation as human food.

MATERIALS AND METHODS

Collection of cassava leaves

In June, 2012, tender cassava leaves, the first matured up to leaf position five were harvested from three species of cassava, bitter (*Manihot utilissima*), sweet (*Manihot dulcis*) and wild (*Manihot glaziovii*). Varieties named “Seruruseke” (5280), ISAR 1961 and “Igicucu” were chosen for sweet, bitter and wild, respectively. In order to minimize the effects of age, environment and soil type on chemical composition, leaves samples of the same age were selected from the same field, Rwanda Agricultural Board (RAB)’s field at the Karama Research Station, in Bugesera District of Eastern Province of Rwanda.

Sample preparation

Samples were collected in the field and transported in closed polyethylene bags, which were stored in a cool box containing ice. Each sample was divided into two portions, first portion was analyzed in fresh condition and for the second portion analysis was done after blanching and drying. Blanching was done by submersion in boiling water for 4-5 min, and then immediately cooled in tap water at ambient temperature as described by Kendall et al. (2010).

Three different preparation procedures were conducted, namely: (1) Un-dried (UND) (2) dried before pounding (DBP) and (3) dried after pounding (DAP) leaves. Pounding was done using wooden mortar and pestle, while drying was done using a tunnel solar dryer at Sokoine University of Agriculture. The products obtained by the three different preparation procedures (Figure 1) were chemically analyzed, un-boiled and boiled for 15 and 30 min. Moisture, cyanide, protein and minerals (Ca, Fe, K, P and Zn) were determined. The first four analyses were conducted at Sokoine University of Agriculture laboratories, while vitamins (Ascorbic acid and β-carotene) analyses were done at the Tanzania Food and Drug Authority (TFDA), in Dar-Es-Salaam. All chemical analyses were carried out in quadruple.

Drying procedures

After blanching, pounded and un-pounded leaves from bitter, sweet and wild cassava were dried using a tunnel solar dryer. Temperatures inside the dryer were recorded at 8 a.m., noon and 8 p.m. each day, averaging 38°C. The complete drying was when the samples were dried until they became brittle. The dried samples were immediately packed in plastic materials, sealed and transported to laboratories, in opaque cartons to avoid light effect before analysis.

Cooking procedures

The cooking consisted of boiling for 15 and 30 min, in distilled water (1:2) and (1:9) respectively for un-dried and dried samples as volume of sample by volume of water, in stainless steel and without cover. The dried samples were first soaked in water for about 5 min before starting the fire. Un-dried and cooked samples were kept frozen before analysis.

Cyanide (HCN) and nutrients determination

Cyanide (HCN) levels in the samples were determined by alkaline titrating method as described by AOAC (1995), official method 915.03B. Moisture content of samples was determined as outlined by AOAC (1995), official method 934.01. For minerals, sample ashes and solutions were obtained respectively by official methods 965.09 and 982.23 described by AOAC (1995). Total phosphorus (P) was obtained using ascorbic acid blue color procedure and by reading the absorbance at a wavelength of 884 nm on a UNICAM 5625 UV/visible spectrometer (Okalebo et al., 1993). Calcium (Ca) and potassium (K) were measured by flame photometry, reading their absorbance at 422.7 and 766.5 nm respectively on a Cole-Parmer instrument, model 2655-00 Digital flame Analyzer. Iron (Fe) and zinc (Zn) were determined by reading their absorbance at 248.3 and 213.9 nm, respectively on a UNICAM 919 Atomic Absorption Spectrometer (AAS) using Hollow Cathode lamps (Okalebo et al., 1993). Crude protein content was determined by using the micro-Kjeldahl method (AOAC, 1995), official method 920.87. Vitamin C (ascorbic acid) content was determined as outlined by ISO (1984), method 6557/2. B-carotene was measured using a high performance liquid chromatography (HPLC), equipped with a Photodiode Array (PDA) detector fitted with a 436 nm wavelength. For sample preparation, aliquots were extracted by solvent n-Hexane (Priadi et al., 2009; Tee Siong and Lam, 1992). Further extraction and clean-up was done using a dispersive Solid Phase Extraction (dSPE) technique as described in AOAC (2007), official method 2007 0.1.

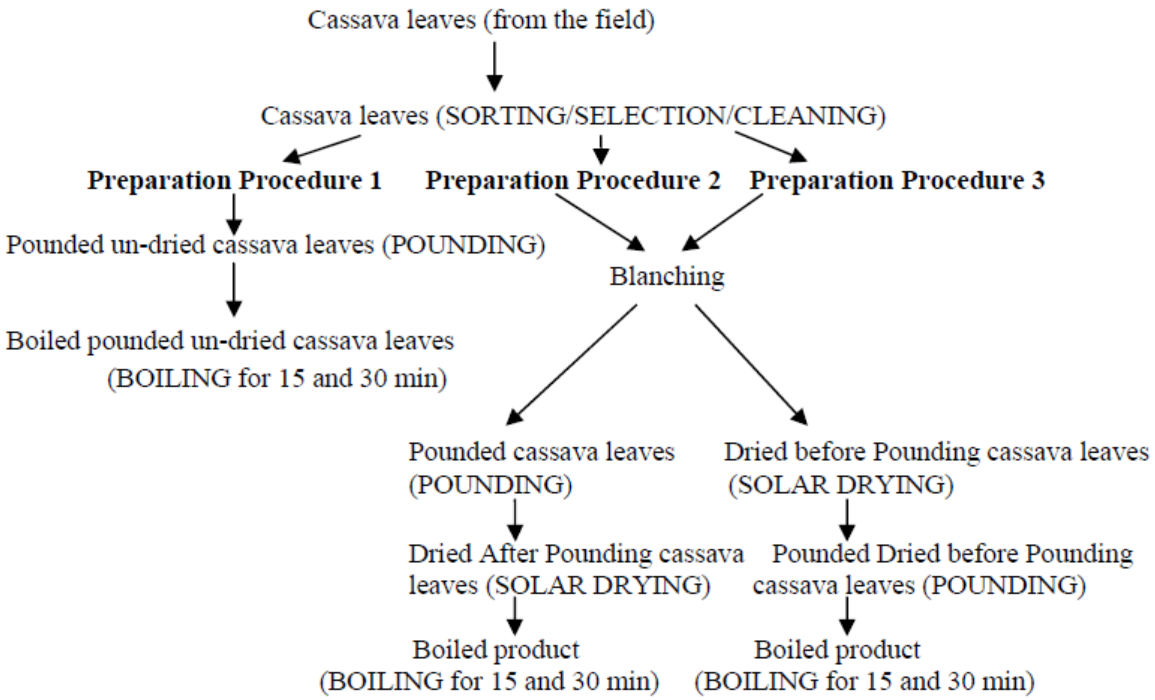

Figure 1. Flow diagram illustrating preparation procedures of cassava leaves.

Statistical data analysis

Data from the results of chemical analyses of samples were subjected to statistical analysis, using SAS 9.2 (SAS Institute, 2008). Kolmogorov-Smirnov test was first carried out to assess the normality of the data (Kutner et al., 2005). Multiple ways (species with three levels specifically bitter, sweet and wild, processing methods with three levels namely un-dried, dried before pounding and dried after pounding, boiling time with 2 levels, that is, 15 and 30 min) analysis of variance (ANOVA) was applied after assuming the normal distribution of the data. Where the treatments had statistical significant effect on the response variables of interest, Fisher's least significant differences (LSD) test was used to separate the means. The treatments were judged statistically significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

Cyanide, ascorbic acid, β-carotene, protein, iron, calcium, phosphorus, potassium and zinc were chemically determined in cassava leaves from three species (bitter, sweet and wild). Overall effect of species and processing procedures on contents are shown in Table 1. From the results in the table, it was noticeable that for un-dried and dried samples, before boiling, wild species had the highest concentrations in all determinations (cyanide, ascorbic acid, β-carotene, protein, iron, calcium, phosphorus, potassium and zinc). Bitter species had also high protein content as the wild. Sweet species was less concentrated in cyanide, β-carotene, crude protein, calcium, phosphorus and potassium than wild and bitter. Bitter and sweet species had similar concentrations of ascorbic acid and iron. Bitter species was less concentrated in zinc than wild and sweet.

The presence of cyanide in all the studied leaves confirmed the earlier reports that all cassava cultivars contain cyanogenic glucoside, in a wide variation according to varieties (CIAT, 2007). The levels of cyanide in fresh leaves of the studied species were 1905, 1480 and 2179 mg HCN/kg respectively, for bitter, sweet and wild. The values were in the ranges reported by earlier researchers, from 189 to 2466 mg HCN/kg fresh weight basis by Fukuba et al. (1982) and 800 to 3200 mg HCN/kg dry matter by Ravindran (1995). The high values in the studied leaves were not surprising as cyanide is known to be distributed throughout the cassava plant, but with highest levels in leaves (Etonihu et al., 2011). However, depending on the varieties, moderate and low cyanide content cassava leaves have been discovered by Burns et al. (2012). As continued consumption of high dietary cyanide has been linked with a number of chronic health disorders (Nhassico et al., 2008; CCDN, 2007), it is evident that, cassava leaves in the present study need to be properly processed to reduce their cyanide before consumption by humans.

Nutrients varied significantly ($p< 0.05$) according to cassava species. Sarkiyayi and Agar (2010) revealed significant differences in protein and mineral contents when investigating sweet and bitter roots and it may be the same phenomenon in the leaves. In un-dried samples, the average values of crude protein (35-36%), iron (230-278 mg/kg), calcium (7373-8822 mg/kg), phosphorus (4413-4907 mg/kg), potassium (15110-17119 mg/kg) and

Table 1. Mean levels of cyanide and selected nutrients of cassava leaves according to species and processing procedure.

Parameter		Cyanide (mg/kg)	Vitamin (mg/100g)		Crude protein (%)	Mineral (mg/kg)				
PP	**CS**	**HCN**	**AA**	**B-C**	**CP**	**Fe**	**Ca**	**P**	**K**	**Zn**
	Bitter	1905.0±25.0^{b}	8.41±0.03^{b}	51.2±0.5^{b}	36.6±0.5^{a}	230.4±1.6^{b}	7441.0±60.3^{b}	4607.7±48.8^{b}	16309.9±102.9^{b}	64.2±0.7^{c}
UND	Sweet	1480.5±18.9^{c}	8.39±0.02^{b}	40.6±0.3^{c}	35.2±0.8^{b}	230.4±1.3^{b}	7373.5±63.3^{c}	4413.3±45.6^{c}	15110.4±116.8^{c}	67.0±0.6^{b}
	Wild	2179.7±29.1^{a}	13.27±0.09^{a}	80.4±0.3^{a}	36.6±0.2^{a}	278.3±2.1^{a}	8222.5±61.8^{a}	4907.4±50.2^{a}	17119.9±109.5^{a}	76.2±0.7^{a}
	Bitter	562.8±24.2^{b}	0.00075±0.00^{b}	43.7±0.8^{b}	36.8±0.7^{a}	222.5±0.6^{b}	6956.1±65.0^{b}	4590.4±43.5^{b}	15520.6±139.4^{b}	61.9±0.7^{c}
DBP	Sweet	467.8±27.6^{c}	0.00075±0.00^{b}	39.8±0.2^{c}	35.1±0.9^{b}	221.8±1.9^{b}	6587.0±60.3^{c}	4212.0±49.7^{c}	14358.1±150.2^{c}	65.5±1.2^{b}
	Wild	873.3±27.3^{a}	0.00090±0.00^{a}	65.9±0.2^{a}	36.1±0.1^{a}	243.0±0.3^{a}	8070.2±62.1^{a}	4877.4±48.6^{a}	16157.4±133.0^{a}	73.2±0.8^{a}
	Bitter	413.6±21.7^{b}	0.00070±0.00^{b}	39.1±0.1^{b}	36.6±0.1^{a}	215.2±0.1^{b}	6360.2±68.7^{b}	4454.6±48.6^{b}	14693.3±156.2^{b}	59.7±1.3^{c}
DAP	Sweet	352.4±23.6^{c}	0.00070±0.00^{b}	29.1±0.0^{c}	35.2±0.1^{b}	215.3±0.2^{b}	6124.7±69.9^{c}	3957.2±52.3^{c}	12922.2±151.0^{c}	62.1±0.2^{b}
	Wild	684.9±21.6^{a}	0.00080±0.00^{a}	63.5±0.2^{a}	36.3±0.1^{a}	228.9±0.5^{a}	7898.8±63.6^{a}	4607.2±48.7^{a}	15765.7±118.7^{a}	69.5±1.6^{a}

Values are means and SE of nine independent determinations, dry matter basis in quadruple. Means within columns superscript by similar letter are not significantly different from each other ($P < 0.05$) by Fisher's least significant difference (LSD). PP = Processing procedures, CS = cassava species, HCN = hydrogen cyanide, AA = ascorbic acid, β-C = β-carotene, CP = crude protein, Fe = iron, Ca = calcium, P = phosphorus, K = potassium, Zn = zinc, UND = un-dried, DBP = dried before pounding and DAP = dried after pounding

zinc (64-76 mg/kg) agreed with values in earlier reports (Dada and Owuru, 2010; Ravindran, 1995). Values of β-carotene (406-804 mg/kg) were in agreement with values mentioned by Priadi et al. (2009). Considering these nutrient levels, it can be said that cassava leaves are good source of β-carotene, protein and minerals and similar observation has been written by Achidi et al. (2005) and Ayodeji (2005). Mulokozi et al. (2007) and Akinwale et al. (2010) noticed also the potential contribution of cassava leaves, especially in vitamin A and suggested to properly prepare them for more profit from their present nutrients.

The cassava leaves were differently processed before cooking: (1) "Un-dried (UND)", (2) "Dried before pounding (DBP)" and (3) "Dried after pounding (DAP)". DBP and DAP leaves were blanched before solar drying, principally for inactivating potentially deleterious enzymes. The leaves were dried to brittle and on average, water content of the leaves varied from 83.5% for fresh to 4.7% for dried. As it is mentioned by James and Kuipers (2003), green vegetables contains less sugar, and thus, they can be dried to brittle and water content, 4-8%, depending on the type of vegetable. Un-dried samples were more concentrated in cyanide, ascorbic acid, β-carotene, iron, calcium and potassium than dried, but crude protein seemed not to be sensibly affected by drying. Dried before pounding and dried after pounding samples were the first in protein and second in iron and potassium. Dried un-pounded (DBP) samples retained more cyanide and nutrients than the dried pounded leaves (DAP). Drying reduced deeply the ascorbic acid content of the samples. The differences between un-dried and dried leaves were due to the combination effects of blanching and drying, because before drying leaves were blanched. It has been revealed that blanching and drying reduce the poisonous compound, cyanide, but unluckily accompanied by nutrients losses (Oguche Gladys, 2011; Anhwange et al., 2011; Eze, 2010; Abah Idah et al., 2010; Udofia et al., 2010). From the same table (Table 1), it was observable that pounding promoted cyanide and nutrients removal. The decrease, probably due to leaching or solubility in evaporated or drained water, was facilitated by small sized particles of dried after pounding products.

After the solar drying, the residual cyanide was still high (684-873 mg HCN/kg dry matter). An additional treatment was necessary for safety of the foodstuffs. Cooking by boiling in water is well known to reduce sensibly cyanide (Gernah et al., 2012; Ubi et al., 2008). Therefore, the UND, DBP and DAP were boiled, in distilled water, using stainless materials, for 15 and 30 min. In addition to cyanide reduction, findings in this study showed a significant ($p < 0.05$) decrease in protein, vitamins and minerals with cooking time and it has been the same observation in earlier study of Gernah et al. (2012). The concern was to assess a state of the cooked cassava leaves in regards to cyanogens and nutrients. Means of cyanide, ascorbic acid, β-carotene, protein, iron, calcium, phosphorus, potassium and zinc of the vegetables,

Table 2. Mean levels of cyanide and selected nutrients of boiled cassava leaves for 15 minutes.

Parameter		Cyanide (mg/kg)	Vitamin (mg/100g)		Crude protein (%)	Mineral (mg/kg)				
PP	CS	HCN	AA	B-C	CP	Fe	Ca	P	K	Zn
UND	Bitter	571.2±5.6^{b}	5.78±0.33^{b}	34.1±1.9^{b}	36.8±0.7^{b}	144.7±0.4^{b}	4230.4±11.9^{c}	3644.0±30.6^{c}	8917.0±87.9^{b}	45.8±0.8^{c}
	Sweet	501.9±1.4^{c}	5.39±0.05^{c}	33.1±2.6^{c}	35.5±0.4^{c}	142.5±1.4^{c}	4622.7±34.1^{b}	3872.9±33.9^{b}	8905.2±149.4^{c}	58.8±0.9^{b}
	Wild	696.5±3.3^{a}	11.49±0.04^{a}	49.1±3.1^{a}	36.9±0.8^{a}	210.4±1.8^{a}	6729.3±19.8^{a}	4533.0±31.6^{a}	8984.3±119.6^{a}	68.7±0.8^{a}
DBP	Bitter	532.8±4.2^{b}	-	35.9±0.2^{b}	36.8±0.7^{b}	92.5±0.6^{c}	4186.2±25.0^{c}	3199.4±23.5^{b}	8520.6±139.4^{b}	45.7±2.3^{c}
	Sweet	467.8±4.6^{c}	-	33.0±4.8^{c}	36.7±0.2^{b}	97.8±1.9^{b}	4487.0 ±20.3^{b}	3212.0±20.7^{b}	8858.1±150.2^{a}	58.1±0.2^{b}
	Wild	543.3±4.3^{a}	-	39.8±0.2^{a}	37.2±0.9^{a}	113.0±0.3^{a}	6370.2±32.1^{a}	4346.3±33.4^{a}	8857.4±133.0^{a}	63.5±1.6^{a}
DAP	Bitter	303.6±3.7^{b}	-	34.2±0.1^{b}	35.3±0.2^{b}	92.2±0.1^{c}	4160.2±28.7^{b}	3177.4±28.6^{b}	8493.3±156.2^{c}	45.1±0.1^{c}
	Sweet	292.4±3.6^{c}	-	29.1±0.1^{c}	35.2±0.1^{b}	95.3±0.3^{b}	4424.7±16.9^{b}	3157.2±42.3^{b}	8822.2±151.0^{b}	58.3±0.2^{b}
	Wild	314.9±3.6^{a}	-	35.5±0.2^{a}	36.7±0.1^{a}	112.9±0.5^{a}	6298.8±36.6^{b}	4307.2±38.7^{a}	8855.7±118.7^{a}	86.3±0.1^{a}

Values are means and SE of nine independent determinations, dry matter basis in quadruple. Means within sub-columns superscript by similar letter are not significantly different from each other ($P < 0.05$) by Fisher's least significant difference (LSD). PP = processing procedures, CS = cassava species, HCN = hydrogen cyanide AA = ascorbic acid, βC = β-carotene, CP=Crude protein, Fe = iron, Ca = calcium, P = phosphorus, K = potassium, Zn = zinc, UND = un-dried, DBP = dried before pounding and DAP = dried after pounding.

boiled for 15 and 30 min are given respectively in Tables 2 and 3.

After boiling for 15 min (Table 2), depending on species and processing procedure, the residual cyanide levels ranged from 209 to 696 mg HCN/kg and remained high so that an extension of cooking time was highly indispensable. After boiling for 30 min (Table 3), the remaining levels of cyanide, across species and processing procedures, varied from 32 to 50 mg HCN/kg. From Tables 2 and 3, processing procedure that excluded blanching (UND) was more effective in removing cyanide by heating than those procedures that included blanching (DBP and DAP). This was attributed to the action of endogenous linamarase on cyanogenic glucosides, following the intimate contact in the finely-divided tissues, during pounding, between linamarin and the hydrolyzing enzyme, linamarase, which promotes rapid breakdown of cyanogens glucosides into a free form, hydrogen cyanide (HCN) (White et al., 2003), while blanching inactivated enzymes in the dried samples and limited easier hydrolysis of cyanogenic glucosides into hydrogen cyanide. The hydrogen cyanide is known to be easily removed by heat during boiling. Similar to drying, after boiling, it was visible that pounding and then drying (DAP) improved cyanide reduction. The reason may be the same for drying, small sized particles of boiled products.

Cassava leaves as a safe human food

After cooking for 30 min, moisture content of the called "relishes" was on average 87%. Therefore, one kilogram (dry weight basis) is equivalent to about 4 kg of the cooked vegetable as it is eaten (relishes). Under normal circumstances, this volume is shared by many persons in one meal considering an adult person can eat up to 100-200 g of the vegetable relish. Furthermore, different studies reported that an acute oral lethal dose of hydrogen cyanide (HCN) is proportional to body weight (WHO, 2004). But a large variation of the doses showed a lack of precision. For example, levels ranging from 30-210 mg of HCN for a 60 kg adult have been recorded by Montgomery (1980). Committee of experts in codex standards concluded that a cyanide level of up to 10 mg HCN/kg of cassava flour is not associated with acute toxicity (FAO/WHO, 1993) and the level became recommended by FAO/WHO (1991) as safe for human foods. Therefore, the cassava leaves in this study, un-dried and solar dried, after being boiled for 30 min, can be said to be safe for human consumption in regards to cyanide toxicity, based on the acute oral lethal doses, but also by considering that the quantities of green vegetables are usually small by serving, as a side relish for the starchy based food. Besides, relish from the leaves was found as source of protein, and the nutrient is known to be helpful in cyanide human body detoxification (Nhassico et al., 2008; CCDN, 2007).

Potential contribution of cassava leaves to human nutrition

For the nutrients, the nine vegetable relishes

Table 3. Mean levels of Cyanide and selected nutrients of boiled cassava leaves for 30 minutes.

Parameter		Cyanide (mg/kg)	Vitamin (mg/100g)		Crude Protein (%)	Mineral (mg/kg)				
PP	**CS**	**HCN**	**AA**	**B-C**	**CP**	**Fe**	**Ca**	**P**	**K**	**Zn**
UND	Bitter	35.4±2.0^{b}	0.24±0.01^{b}	33.0±1.0^{b}	33.7±0.6^{b}	199.4±1.4^{b}	3939.8±27.8^{c}	3564.9±16.5^{c}	8849.3±102.6^{b}	45.4±0.4^{c}
	Sweet	32.8±1.5^{c}	0.20±0.03^{c}	32.6±0.4^{c}	33.8±0.4^{b}	182.7±0.8^{c}	4391.4±24.7^{b}	3795.5±28.2^{b}	8834.1±159.1^{b}	57.9±0.1^{b}
	Wild	40.7±1.5^{a}	0.30±0.01^{a}	43.2±1.9^{a}	34.3±0.2^{a}	219.6±2.5^{a}	5939.3±27.5^{a}	4321.7±23.2^{a}	8960.3±173.5^{a}	68.4±1.2^{a}
DBP	Bitter	47.7±1.1^{b}	-	33.5±0.6^{b}	35.8±0.1^{a}	144.5±2.8^{b}	3509.7±26.3^{c}	3120.9±35.5^{c}	8216.6±124.3^{c}	45.1±0.2^{c}
	Sweet	45.2±0.8^{c}	-	32.6±0.8^{c}	35.0±1.2^{b}	143.9±1.4^{c}	3654.3±28.3^{b}	3200.4±23.6^{b}	8521.8±169.7^{b}	54.4±0.7^{b}
	Wild	50.4±0.4^{a}	-	37.7±0.3^{a}	35.9±0.6^{a}	205.9±3.5^{a}	5436.0±25.6^{a}	4166.9±4.2^{a}	8861.1±180.9^{a}	63.0±1.2^{a}
DAP	Bitter	38.0±2.9^{b}	-	33.2±0.2^{b}	35.3±0.9^{a}	87.1±3.6^{c}	3221.6±33.3^{c}	2982.9±25.0^{b}	7840.7±181.6^{b}	44.5±0.7^{c}
	Sweet	36.4±1.8^{c}	-	29.0±1.8^{c}	35.4±0.5^{a}	95.0±0.6^{b}	3400.2±22.6^{b}	2737.8±25.1^{c}	7008.9±171.6^{c}	51.9±0.4^{b}
	Wild	41.8±2.1^{a}	-	34.3±0.1^{a}	35.0±0.4^{b}	103.9±1.1^{a}	4888.4±18.9^{a}	3898.3±16.9^{a}	8746.5±168.0^{a}	61.7±0.6^{a}

Values are means and SE of nine independent determinations, dry matter basis in quadruple. Means within sub-columns superscript by similar letter are not significantly different from each other ($P < 0.05$) by Fisher's Least Significant Difference (LSD). PP = Processing procedures, CS = cassava species, HCN = hydrogen cyanide AA = ascorbic acid, βC = β-carotene, CP = crude protein, Fe = iron, Ca = calcium, P = phosphorus, K = potassium, Zn = zinc, PM = processing method, SP = species, UND = un-dried, DBP = dried before pounding and DAP = dried after pounding.

retained appreciable levels of the studied nutrients, except vitamin C, for which levels were too small to be considered as traces in relishes from dried and cooked cassava leaves. The severe reduction of ascorbic acid may be related to the fact that it is thermo-labile at mild heating and very sensitive to blanching, drying and cooking (Faber and Van Jaarsveld, 2007). To understand the contribution of the relishes to human nutrition, their content levels of β-carotene, protein, iron, calcium, phosphorus, potassium and zinc were compared with Recommended Dietary Allowances (RDAs). For β-carotene, because the body converts all dietary sources of vitamin A into retinol, it is explained as retinol activity equivalent (RAE) and believing that 1 µg of retinol is equal to 6 µg of β-carotene (Food and Nutrition Board, 2001), the β-carotene mean values were calculated into RAE before being compared to RDA. Results of the comparison are shown by Table 4.

From the Table 4, the amounts in grams (dry matter basis) of relish from un-dried (UND) leaves (128, 50, 190, 205, 202, 263, and 14 g) to meet respectively protein, iron, calcium, phosphorus, potassium, zinc and β-carotene RDAs, were less than the amounts of relishes from DBP (128, 60, 208, 228, 210, 272 and 15 g), and DAP (132, 105, 228, 249, 228, 287 and 16 g), needed to meet the respective nutrients (protein, iron, calcium, phosphorus, potassium, zinc and β-carotene) RDAs.

The results showed that relish from un-dried leaves (UND) provides more nutrients than relish from dried leaves. This was attributed to blanching and drying, indispensable treatments for quality and storability (Oguche Gladys, 2011; Anhwange et al., 2011; Eze, 2010). Moreover, comparing the dried samples, drying before pounding (DBP) procedure provides more nutrients than pounding before drying treatment (DAP), but the latter contains less cyanide and then is safer for human consumption. B-carotene and iron are adequately contributed by the cassava leaves, considering the slighter required quantities of the greens to meet their RDAs (Table 4).

Conclusion and recommendations

Cassava leaves of bitter, sweet and wild species, when un-dried or solar dried, have potential to contribute to vitamin A, protein and mineral requirements. Vitamin C is very low in cooked (un-dried and dried) cassava leaves that a complement vitamin C rich-food is necessary to accompany cassava leafy meal. Leaves from wild species are the richest in nutrients, followed by bitter while sweet is the least. Fresh (un-dried) leaves give the more safe and rich foods, but for preservation purposes, solar drying is efficient, and pounding cassava leaves before they are dried is a more recommended drying method for cyanide reduction. Cyanide levels are significantly different in leaves from different cassava species, but after sufficient cooking, the difference is small that vegetable relishes from all the cassava species can be judged to be safe for human consumption. Blanching, drying and prolonged cooking treatments (30 min) reduce sensibly cyanide levels

Table 4. Average levels of nutrients in processed and cooked (30 min) cassava leaves and required amount of the relishes to meet the RDAs.

PP	CP (%)	Fe (mg/kg)	Ca (mg/kg)	P(mg/kg)	K (mg/kg)	Zn (mg/kg)	B-C (µgRAE/g)
UND	35.3±0.4	200.7±3.9	4756.5±59.9	3893.0±37.2	8881.2±180.4	56.6±1.1	61.5
DBP	35.5±1.3	164.1±2.5	4199.4±24.8	3495.5±28.8	8532.4±162.5	54.8±0.8	58.1
DAP	33.3±1.0	95.3±1.4	3836.7±32.6	3205.3±33.5	7864.7±165.4	52.5±1.3	54.4
RDA(mg)	45,000	10	800	800	1800	15	900 (µg RAE)
Required amount of the vegetable relishes (g) to meet the RDAs (dry matter basis)							
UND	128.5	50.2	190.5	205.4	202.6	263.6	14.6
DBP	128.3	60.7	208.0	228.7	210.5	272.3	15.4
DAP	132.3	105.2	228.6	249.6	228.6	287.7	16.5

Values are means and SE of six independent determinations, dry matter basis in quadruple. CP = crude protein, Fe = iron, Ca = calcium, P = phosphorus, K = potassium, Zn = zinc, PP = processing procedures, UND = un-dried, DBP = dried before pounding, DAP = dried after pounding, RDAs = Recommended Dietary Allowances by day and RAE = retinol activity equivalent.

(32-50 mg HCN/kg dry matter basis), but not at recommended level for human foods (10 mg HCN/kg dry matter basis). However, considering the small quantities by serving of green vegetables as side food, the protein level in cassava leaves, important nutrient in cyanide human body detoxification, and the acute oral lethal doses of hydrogen cyanide by bodyweight, the cassava leaves food can be said to be safe for human consumption. However, it is not advisable to consume cassava leaves as an everyday vegetable relish or in large quantities. In addition, cassava leaves meals may be limited for lower body weights such as children. The frequency may be reduced by promoting other greens such as amaranths, spinach and cabbage, even in arid areas, where cassava leaves are highly utilized as human food because other leafy vegetables cannot grow well in the present conditions. Cassava varieties with low levels of cyanide in leaves should be released for leaves consumption purposes to alleviate nutrient deficiencies, especially β-carotene and iron.

In general, amounts of nutrients retained after cooking un-dried and dried cassava leaves (after 30 min) are significant that cassava leaves as food are judged to contribute nutritionally to human health, especially vitamin A and iron.

Protein content is also of interest, and as cassava leaves are affordable by even poor people whose access to protein rich-foods such as milk, meat and fish is hard, the leaves can be helpful. But time of cooking may be extended to at least 30 min to improve reduction of cyanide level in the cassava leaves relishes.

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Biogenic amines and microbiological profile of egyptian cheeses

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Cheeses are among those high-protein-containing foodstuffs in which enzymatic and microbial activities cause the formation of biogenic amines (BAs) from amino acids decarboxylation. Most of the methods for amine determination in these products involve acid extraction followed by a liquid-liquid purification step to selectively separate amines and amino acids. This study aimed to describe the development of biogenic amines in Egyptian cheeses during ripening and storage regimes. Biogenic amines content in Mish, Ras and Blue cheeses were 270-1300, 340-980 and 210-700 mg/kg, respectively. The dominant biogenic amines were different. This work confirms that the main biological feature influencing amines formation is the extent of growth of microorganisms, like *Enterococci*, characterized by decarboxylase activity. It is important to note that the presence of biogenic amines due to the activities of these microorganisms is maintained within safe levels. In Egypt, reports dealing with the Egyptian cheeses (Mish, Ras and Blue) are scanty. So, the present work was carried out to fill the gap in our knowledge on its microbiological and biochemical features, focusing on hygiene and consumer health aspects.

Key words: Biogenic amines, food safety, proteolysis, ripening, *Enterococci* spp.

INTRODUCTION

Milk and milk products are very important in human nutrition and, among them; cheese is considered a good source of proteins, vitamins and minerals. However, cheese is one of the most fermented foods commonly associated with biogenic amines (BAs) contamination. These compounds are basic nitrogenous compounds formed by series of microorganisms, mainly by decarboxylation of amino acids or "*in vivo*" also by de-amination and trans-amination of aldehydes and ketones (Loizzo et al., 2012, 2013). Biogenic amines are compounds commonly present in living organisms in which they are responsible for many essential functions. They can be naturally present in many foods such as fruits and vegetables, meat, fish, chocolate and milk, but they can also be produced in high amounts by microorganisms through the activity of amino acid decarboxylases (Ten Brink et al., 1990). Excessive consumption of these amines can be of health concern because when there is are no equilibrate assumption in human organism, can generate different degrees of diseases determined by their action on nervous, gastric and intestinal systems and blood pressure (Suzzi and

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Gardini, 2003). Biogenic amines are low molecular weight nitrogenous bases, they were found in fermented foods and cheese (Mohamed et al., 2013).

Also, biogenic amines are low-molecular nitrogenous compounds that are formed in foodstuffs mainly by microbial decarboxylation of the precursor amino acids (Alberto et al., 2002). The importance of observing BAs content lies in potential toxicity to human, mainly when the concentration is up to 100 mg/kg (or up to 100 mg/L). Thus, the presence of BAs significantly influences the food quality and safety (Smit et al., 2005).

The presence of relevant amounts of BAs in cheeses has been documented (Martuscelli et al., 2005; Kung et al., 2007; Pintado et al., 2008; Ladero et al., 2009; Mercogliano et al., 2010). In cheeses, BAs formation is caused by curdling and cheese decarboxylase-positive microorganisms. Histamine (HIS), tyramine (TYR), putrescine (PTR), cadaverine (CAD), spermidine (SPD), spermine (SPR), tryptamine (T), and β-phenylethylamine (PE) are frequently found in these products. Cheese is one of the fermented foods most commonly associated with BAs poisoning; mainly HIS, TYR, PTR and CAD. Indeed, the term ‘‘cheese reaction” to refer to it (Ten Brink et al., 1990). Tyramine and histamine are the most abundant and frequent BAs in cheese (Fernández et al., 2007). Consumption of food containing high levels of BAs is considered undesirable since it can be associated with several toxicological problems such as respiratory distress, headache, hyper- or hypo-tension or allergies (Ladero et al., 2010). These problems are especially severe in consumers with low levels of the enzymes involved in the detoxification system (mono and di-amine oxidases), either by genetic disorders (Caston et al., 2002) or medical treatments (Halász et al., 1994). The content of biogenic amines and polyamines significantly differed according to the technology of ripening. The cheeses unwashed during ripening had much higher contents of all observed amines and polyamines in comparison with the washed-rind cheeses. The mean content of putrescine, cadaverine and tyramine exceeded 100 mg/kg in unwashed-rind cheeses, while the other amines occurred at lower levels. The content of all detected amines was very low in washed-rind cheeses; no tryptamine, phenylethylamine and histamine were found. The effect of storage on the amine formation was not confirmed (Samková et al., 2013).

Physiologically, histamine is one of the most effective BAs; it has vasoactive and psychoactive effects (Repka-Ramirez and Baraniuk, 2002). Moreover, it is the main BAs involved in food poisoning and it is limited in some foodstuffs by law. At non-toxic doses, food borne histamine can cause intolerance symptoms such as diarrhoea, hypotension, headache, pruritus and flushes. Just 75 mg of histamine, a quantity commonly present in some meals, can induce symptoms in the majority of healthy persons with no history of histamine intolerance (Wöhrl et al., 2004).

The ability of microorganisms to decarboxylate amino acid is highly variable. Due to strain-specific, it is important to count decarboxylase-positive microorganisms to estimate; the risk of BAs food content and to prevent BAs accumulation in food products. Presence and accumulation of BAs depends on many factors such as presence of specific bacteria (*Enterococci, Micrococci, Enterobacteriaceae* and *Lactobacilli*) and enzymes, availability of free amino acids, presence of suitable cofactors, that is, pH level, water activity, temperature and salt content, type of cheese, ripening and storage period (Galgano et al., 2001). Some controversial results have been reported on the contribution of *Enterococci* sp. in BAs production in cheeses, and in particular in histamine (Sumner and Taylor, 1989). *Enterococci* have a long history of use as artisanal cultures for preparation of various types of cheeses (Izquierdo et al., 2009), they are sometimes associated with pathogenicity (Khan et al., 2010), can cause endocarditic, bacteraemia, and several infections, as well as multiple antibiotic resistances (Kayser, 2003). Although, the chemical composition and microbiological quality of cheeses in Egyptian markets have been studied extensively, little data is available on the occurrence of biogenic amines in Egyptian cheeses. Therefore, this survey was undertaken to determine the presence of BAs in commercially available cheeses during ripening and storage, also to make an assessment of the health hazard arising from the consumption of these products especially by susceptible individuals. Other studies such as Lorencová et al. (2012) and Buňková et al. (2010) deal with selection and study of microorganism (such as lactic acid bacteria), which are major producer of biogenic amine. However, these works explore the particular biogenic amine production in growth medium, where the concentration of biogenic amine could be biased optimal environment for the bacteria metabolisms. Moreover, some strains of the starter lactic acid bacteria (such as *Lactococcus lactis* subsp. *lactis*) have decarboxylase activity that was observed in model environment of growth broth. Behaviour of these strains has not been investigated in real system of the cheese and can be different in comparison with condition in growth broth.

The objective of our pilot study was to compare the BAs content and other selected parameters in Egyptian cheese and to review hypothesis that the BAs content developed during the ripening and storage period is related to the presence of decarboxylase positive strain of *Enterococci* sp.

MATERIALS AND METHODS

Cheese samples

A total 85, 49 and 44 of Mish, Ras and Blue cheeses samples were

purchased from different Egyptian retail markets and small scale factories. The samples collected were 6-48 months old. They were kept in sterile plastic bags and transported to the laboratory of Food Science Department, Zagazig University (Egypt), then stored at 4 ±1°C until analyzed.

Chemical analysis

Cheeses were analyzed in triplicates for moisture by the oven drying method at 102°C (IDF, 1993), salt by titration with $AgNO_3$, and fat by Gerber method (AOAC, 2002). For pH measurement, grated cheese (10 g) was macerated with 10 mL of distilled water and the pH of the resultant slurry was measured using a digital pH meter (pH 211, Hanna Instruments, Vila do Conde, Portugal). Titratable acidity was determined as g lactic acid/100 g cheese is using the method of AOAC (2002). Total volatile fatty acids and total nitrogen (TN) were determined using the methods of AOAC (2002). All analyses were carried out in triplicates.

Assessment of proteolysis

Water-soluble nitrogen fraction (WSN) of cheese was prepared according to Kuchroo and Fox (1982) and a cheese to water ratio of 1:5 was used. 12% trichloroacetic acid soluble nitrogen-fraction (TCA-SN, that is, NPN) was obtained by mixing equal volumes of water-soluble fraction and 24% (w/w) TCA solution, followed by filtration through a white ribbon filter paper (Schleicher and Schuell, Dassel, Germany). The nitrogen content of both fractions WSN and TCA-SN, respectively, was determined by Kjeldahl method (AOAC, 2002) and expressed as percentage of TN.

Free amino acids and biogenic amines

Free amino acids (FAA) and BAs were assayed according to the method of Krause et al. (1995), modified by Pinho et al. (2001). In brief, a 4 g cheese sample was suspended in 15 mL of 0.2 M aqueous perchloric acid; the mixture was homogenized in an Ultra Turrax blender (Sotel, Warsawa, Poland) for 2 min, then kept in an ultrasonic bath (Heraeus, Osterode, Germany) for 30 min, and finally centrifuged at 4000 xg for 20min. Derivatization was carried out via dansyl chloride, at 70°C per 15 min. The reaction was quenched by placing the vials in an ice bath for 5 min. High performance liquid chromatography (HPLC, Waters 600) was used to dansylamines determination. The system was equipped with delivery system, reverse phase CI8 Nucleosil column 250 x 4 mm, 10 µm packing (Macherey - Naggl). The detection was performed using U.V detector (Waters 486) at wavelength of 254 nm using linear program of 25 min period and 1 ml/min constant solvent flow rate. Data were integrated and recorded using a Millennium Chromatography (Waters, Milford MA 01757). Elution was carried out at a flow rate of 1 mL/ min, using a volumetric gradient of solution A, 9 mM aqueous sodium dihydrogenophosphate, 4% (w/v) dimethyl formamide and 0.1% (w/v) triethylamine (adjusted to pH 6.55 with phosphoric acid), and solution B, 80% (v/v) aqueous acetonitrile. Detection was performed by measuring absorbance at 436 nm. Quantification was carried out based on a mixture of amino acid standards: aspartic acid, glutamic acid, serine, threonine, glycine, alanine, arginine, proline, valine, methionine, isoleucine, leucine, lysine, histidine, tyrosine, cystine, tryptophan and phenylalanine; and biogenic amine standards: ornithine, tryptamine, phenyl ethylamine, putrescine, cadaverine, histamine, tyramine and spermine (Sigma Chemical). All determinations were performed in triplicates (Figure 1).

Microbiological analysis

For each cheese sample, 10 g was weighed and dispersed aseptically in 90 mL of citrate buffer (2%,w/v) and homogenized in a sterile polyethylene bag using a Stomacher (Seward Laboratory Blender Stomacher 400 Lab Blender UK) for 1.5 min. Serial dilutions were made in 0.1% sterile peptone water and all determinations were made in triplicates (Messer et al., 1985). The enumeration of total mesophilic bacteria (Plate Count Agar, Merck, Germany) at 30°C/48 h, total coliform groups (Violet Red Bile Agar, Merck, Germany) at 37°C/48 h, yeasts and moulds (Potato Dextrose Agar, Merck, Germany) at 21°C/7 days, *Lactobacilli* (MRS agar, Merck, Germany), *Lactococcus* sp. (M_{17} agar, Merck, Germany) and *Enterococci* (Azide Dextrose agar, Merck, Germany) at 28°C/48 h (Frank et al., 1993) were performed.

Statistical analysis

The effect of time of ripening on all parameters of proteolysis and on total FAA and BAs content of the cheese was assessed by analysis of variance (ANOVA) using the SPSS 10.0 for Windows software (Liu et al., 2003).

RESULTS AND DISCUSSION

Evaluation of physico-chemical parameters

The chemical compositions of Egyptian cheeses are presented in Table 1. The total solids content of cheese samples varied from 30.5 to 46.5, 47.2 to 58.3 and 41.2 to 48.8% in Mish, Ras and Blue cheeses, respectively. A significant variation of fat content was observed, 17.8-30.4; 33.8-48.3 and 25.3-38.4% in Mish, Ras and Blue cheeses, respectively. The salt content of the cheese samples fell within the range, 6.1-10.5; 5.6-6.8 and 4.5-5.7% in Mish, Ras and Blue cheeses, respectively. Whereas, the pH of cheeses ranged from 4.2 to 5.3; 4.5-5.2 and 4.9-5.8 respectively, which agrees with those reported for good quality Egyptian cheeses (Kebary et al., 1999; Ibrahim and Amer, 2010). Total nitrogen content in cheese samples was slightly higher in Ras cheese as compared to Mish and blue cheeses. Whilst, the water soluble nitrogen was lowest in Blue cheese as compared to other cheeses (Table 1). The WSN/TN ratio showed differences in the degree of ripening of the component cheeses. NPN represented more than 50% of the WSN of the tested cheeses; this may have originated from the component cheeses. WSN and NPN have been classically used as a measure of the extent of secondary proteolysis, that is, formation of small sized peptides (2–20 residues) and free amino acids (Furtado and Partridge, 1988). Total volatile fatty acids showed a significant variation among the tested cheeses, 33.5-55.4; 62.7-92.7 and 45.6-74.5 as 0.1 N NaOH/100 g, in Mish, Ras and Blue cheese, respectively. These variations indicated large differences in quality and degree of ripening in Egyptian cheeses. Production of BAs has frequently been

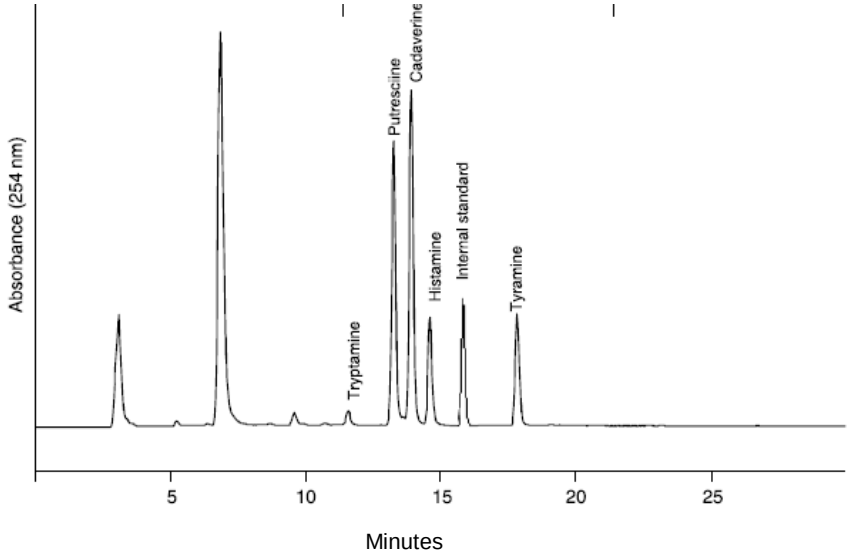

Figure 1. Chromatograms present the areas of biogenic amine standard solution derivatized with incubation at 40°C for 40 min.

Table 1. Chemical composition and proteolysis indices of Egyptian cheese samples ripened/stored at different periods.

Cheese type	Ripening/storage period (mon)	Physico-chemical parameter						Proteolysis indices			
		Total Solid (%)	Fat in solid (%)	Protein in solid (%)	Acidity *	pH	Salt in solid (%)	Total nitrogen (%)	Non-protein nitrogen (%)	Water soluble nitrogen (%)	TVFA**
Mish	6	30.5±2.0^{d}	17.8±2.5^{d}	20.1±1.07^{a}	3.31±0.71^{a}	4.2±0.1^{d}	6.1±1.5^{b}	3.15±0.98^{a}	0.22±0.4^{b}	0.29±0.5^{d}	33.5±2.2c
	12	35.6±2.1^{c}	20.8±2.2^{cd}	18.5±1.15^{b}	3.01±0.65^{b}	4.4±0.1^{cd}	7.2±1.6^{ab}	2.91±0.78^{a}b	0.31±0.6^{a}	0.34±0.6^{cd}	40.5±2.5b
	24	37.0±1.9^{b}	22.3±3.2^{bc}	17.23±1.01^{b}	2.8±0.52^{b}	4.5±0.2^{c}	8.3±2.4^{ab}	2.71±0.73^{b}c	0.33±0.6^{a}	0.41±0.4^{bc}	47.9±2.9a
	36	39.4±2.5^{b}	25.6±5.2^{b}	14.35±0.8^{c}	2.5±0.53^{c}	4.9±0.2^{b}	9.2±2.4^{ab}	2.24±0.56^{cd}	0.36±0.7^{a}	0.49±0.4^{b}	52.3±3.1a
	48	46.5±2.7^{a}	30.4±4.5^{a}	13.33±0.5^{c}	2.2±0.44^{c}	5.3±0.2^{a}	10.5±2.9^{a}	2.19±0.57d	0.39±0.7a	0.55±0.3^{a}	55.4±3.2a
Ras	6	47.2±2.9d	33.8±1.5d	24.88±1.8a	2.2 ±0.43a	4.5±0.2b	5.6±1.4b	3.91±1.29ab	0.19±0.1ns	0.31±0.6ns	62.7±3.5d
	9	50.01±3.1c	37.8±2.0c	24.44±1.3b	2.1±0.42ab	4.6±0.1b	6.7±1.5ab	3.81±1.17a	0.20±0.2ns	0.32±0.5ns	74.0±3.8c
	12	52.5±3.6b	42.5±2.4b	23.6±1.4b	2.0±0.34bc	4.8±0.2b	7.3±1.7a	3.71±1.35b	0.21±0.2ns	0.33±0.5ns	85.8±4.3b
	24	58.3±3.8a	48.3±3.1a	21.05±1.0c	1.85±0.23c	5.2±0.1a	6.8±1.9a	3.31±1.42ns	0.25±0.4ns	0.39±0.7ns	92.7±4.5a
Blue-viened	6	41.2±2.2c	25.3±2.1c	22.64±1.2a	1.9±0.22b	4.9±0.3b	4.5±1.2b	3.55±1.47ns	0.20±0.2b	0.25±0.3b	45.6±2.7c
	9	44.5±2.4b	32.1±2.9b	21.69±1.1b	1.7±0.22c	5.1±0.5b	4.8±1.5ab	3.2±1.36ns	0.21±0.3ab	0.24±0.4b	66.6±3.7b
	12	48.8±2.8^{a}	38.4±3.6^{a}	20.41±1.1^{c}	2.8±0.34^{a}	5.8±0.2^{a}	5.7±1.7^{a}	3.4±1.38^{ns}	0.24±0.4^{a}	0.34±0.5^{a}	74.5±3.9a

Averaged data of analyzed cheese samples in triplicate; Mean value ± a standard deviation ***** Acidity is expressed as lactic acid. ** Total volatile fatty acids is expressed as 0.1 N NaOH/100 g cheese.

Table 2. Levels of biogenic amines (mg/100 g cheese) of Egyptian cheese samples ripened/stored at different periods.

Cheese type	Ripening/storage period (month)	Biogenic amines (mg/100 g)								
		TYR	T	PTR	HIS	CAD	SPD	SPR	PE	Total
Mish	6	6±0.62^{e}	4±0.51^{d}	4±0.45^{d}	9±0.69^{e}	3±0.3^{e}	1±0.05^{d}	Nd^{c}	Nd^{d}	27±2.2
	12	12±1.5^{d}	10±0.85^{c}	10±0.85^{c}	14±0.98^{d}	10±0.5^{d}	1±0.11^{d}	Nd^{c}	Nd^{d}	57±3.2
	24	14±0.42^{c}	17±1.54^{b}	18±1.5^{b}	27±2.4^{c}	18±1.72^{c}	2±0.21^{c}	1±0.08^{b}	4±0.53^{c}	101±4.3
	36	15±0.46^{b}	21±1.79^{a}	19±1.7a^{b}	29±2.3^{b}	20±2.1^{b}	3±0.42^{b}	1±0.09^{b}	7±0.64^{b}	115±4.6
	48	19±1.55^{a}	22±2.15^{a}	20±2.16^{a}	31±2.5^{a}	22±1.97^{a}	4±0.63^{a}	2±0.16^{a}	12±1.15^{d}	132±5.3
Ras	6	3±0.28^{d}	10±0.93^{b}	6±0.42^{d}	12±0.96^{d}	Nd^{d}	1±0.08^{b}	Nd^{b}	3±0.19^{b}	34±2.6
	9	4±0.54^{c}	11±1.05^{c}	8±0.62^{c}	14±0.91^{c}	8±0.71^{c}	1±0.11^{b}	Nd^{b}	3±0.21^{b}	49±2.8
	12	5±0.61^{b}	13±1.13^{b}	13±0.54^{b}	23±2.05^{b}	13±1.26^{b}	Nd^{c}	1±0.08^{a}	5±0.41^{a}	73±3.7
	24	14±1.07^{a}	20±1.86^{a}	16±1.25^{a}	26±2.4^{a}	20±2.1^{a}	2±0.21^{a}	Nd^{b}	Nd^{c}	98±4.1
Blue-viened	6	Nd^{c}	11±0.45^{c}	1±0.15^{c}	4±0.34^{c}	4±0.22^{c}	Nd^{b}	Nd^{b}	1±0.11^{c}	21±2.1
	9	1±0.1^{b}	15±0.87^{b}	2±0.31^{b}	9±0.94^{b}	7±0.65^{b}	Nd^{b}	Nd^{b}	2±0.25^{b}	36±2.6
	12	8±0.72^{a}	17±1.26^{a}	9±0.93^{a}	14±1.26^{a}	11±1.13^{a}	3±0.41^{a}	2±0.17^{a}	6±0.62^{a}	70±3.5

Averaged data of analyzed cheese samples in triplicates, nd: not detected.

referred to as the proteolytic activity of microorganisms present in cheese during manufacture and ripening. Increases in the non-protein nitrogen fractions (WSN and NPN) often means level increase of free amino acids, which are precursors of BAs.

Amino acids and BAs

BAs content of cheese can be extremely variable and depends on the type of cheese, the ripening time, the manufacturing process and the microorganisms present (Ordonez et al., 1997). The Egyptian cheeses (Mish, Ras and Blue) examined confirmed this variability in the total content of BAs ranging from 21.0 to 130.0 mg/100 g cheese (Table 2 and Figure 2). There are significant differences among contents of the eight BAs assayed. Only the Mish cheese contained more than 100 mg/100 g cheese of the total BAs, as affected by increasing the storage period. According to Taylor (1985), the threshold of risk is 100 mg/kg total amines of cheese, if ingestion is associated with such potentiating co-factors as amine oxidase-inhibiting drugs or alcohol, or else if there are pre-existing gastrointestinal diseases (Stratton et al. 1991). Production of BAs in cheese has often been associated with non-starter lactic acid bacteria and *Enterobacteriaceae* (Joosten and Northolt, 1987), so it may be a toxicological risk associated with consumption of raw milk cheese, especially by sensitive individuals. Spanjer and van Roode (1991) suggested that the total concentration of tyramine, histamine, putrescine and cadaverine in cheese should not exceed 900 mg/kg DW^{-1}, but no upper limit for BAs in cheese has been legally enforced.

Even if no significant differences were observed in the final amounts of BAs in Blue and Ras cheeses, the dynamics of accumulation were not the same. Overall, histamine was the most prevalent amine, being found in all analyzed cheese samples. It was followed by T (98%), PTR (97%), CAD (95%), TYR (89%), SPD (73%), PE (72%) and SPR (37%). In spite of being the most frequently detected amine, SPR was present at low levels, below 2.5 mg/ 100 g cheese. Spermidine and PE were also detected at low levels (below 4 and 12mg/100 g cheese, respectively). However, HIS, CAD, T, PTR and TYR were detected at levels up to 30, 21, 20, 19 and 18 mg/ 100 g, respectively. HIS was the most prevalent amine, it was found in all analyzed cheese samples (Table 2). Higher means levels were detected for Mish, Ras and Blue cheeses (9-31, 12-26 and 4-14 mg/ 100 g, respectively). HIS levels capable of causing histamine poisoning were detected in all cheese samples. However, taking into account the concomitant presence of polyamines, it is likely that a higher percentage of cheese samples could cause HIS poisoning. TYR was present in 100% of Mish and Ras cheeses and in 60% of Blue cheese. Mish (53%) and Ras cheese (18%) contained TYR at levels capable of causing hypertensive crisis (Komprda et al., 2008). Overall, T was detected sporadically, at lower amounts as compared to HIS. Similar results were observed by Chang et al. (1985). Higher means levels of tryptamine were observed in Mish and Ras cheeses. The toxic threshold of tryptamine is not known (Joosten, 1988). PE, another amine of health significance was detected 100% in Blue, 82% in Ras and 53% in Mish cheese. The prevalence of this amine was

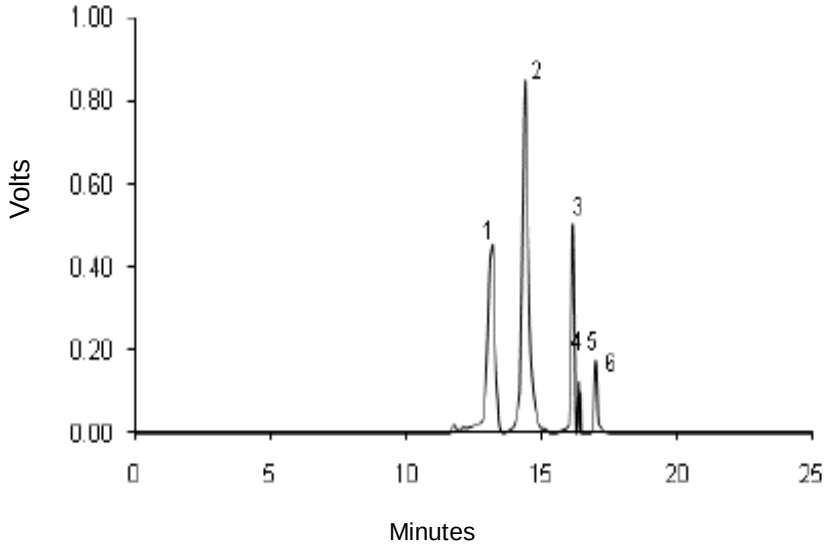

Figure 2. Chromatograms presenting the concentration of histamine, tryptamine, putrescine, tyramine, phenyl ethylamine and cadaverine in Ras cheese.

high, however the levels detected were low (≤12 mg/100 g), below its toxic threshold. The rate of CAD and T accumulation were similar for the traditional Mish and Ras cheeses and that described for the total BAs formation. The accumulation of amines increased remarkably later in the ripening and storage periods. The rate of PE accumulation was similar for Ras and Blue cheeses, whereas HIS and T values were higher in the Ras cheese and reached concentrations of 12 - 26 mg/100 g and 10-20 mg/100 g, respectively.

Similar results were obtained with heat treatment or bactofugation of the milk used for emmental production and had little effect on the TYR content (Krause et al., 1997). The rates of SPR and SPD accumulation were similar for Mish and Blue cheeses, whereas T and HIS were higher in the Mish cheeses and reached concentrations of 4-22 and 9-31 mg/100 g, respectively. According to Halász et al. (1994), Gouda cheese along with Swiss and Cheddar cheeses, which contain high levels of BAs are the most frequently incriminated cheese in histamine poisoning episodes.

Amino acid levels in cheeses types were extremely variable (Table 3). This fact was attributed to an accelerated amino acid release at the manufacturing day, when cheeses were incubated at temperatures favorable for microorganism development and activity (Bütikofer and Fuchs, 1997). Any food with free amino acids, especially tyrosine and phenylalanine, are subject to BAs formation if poor sanitation and low quality foods are used or if the food was subjected to temperature abuse or extended storage time (Schirone et al., 2011).

High variability was observed in pH, acidity, moisture and fat contents of the different analyzed cheese types. Several analyzed samples did not meet the standard of identity and quality established by Egyptian legislation. With regard to quality parameters, pH, moisture and fat content and acidity correlated significantly (P≤ 0.05) with formation and accumulation of some BAs. These results suggest that, among quality parameters evaluated, acidity influenced amine formation in several cheese types. These results are supported by the theory that the formation of BAs is a protective mechanism of bacteria against acidic environments (Maijala, 1994). The presence of micro-organisms with high decarboxylase activity has been reported as the main factor for BAs production in cheese. Moreover, some strains have proteolytic activity, which can affect the accumulation of BAs in cheese (Galgano et al., 2001). For the production of amines, the enzymatic activity of proteases derived from micro-organisms, or from another origin, is important from a qualitative point of view, that is, in relation to the type of amino acids provided to the amino acid decarboxylating microflora. The bacteriological composition of milk could be critical to define the amine profile in cheese; therefore, large amounts of amines in cheese could indicate unsuitability from a hygienic point of view, and the milk used for cheese making. Moreover, the results emphasize the necessity of controlling the indigenous bacterial population responsible for high production of BAs and the use of competitive adjunct cultures is suggested.

Microbiological evaluation

Microbiological analyses of the Egyptian cheeses were examined throughout ripening/storage period (Table 4). Lactic acid bacteria did not show any substantial change

Table 3. Total amount of free amino acids content (mg/g dry weight of cheese) of Egyptian cheese ripened/stored at different periods.

Amino acid	Mish cheese					Ras cheese				Blue cheese		
	Ripening/storage period (mon)											
	6	12	24	36	48	6	9	12	24	6	9	12
Threonine	1.38±0.1	1.36±0.13	1.38±0.13	1.20±0.11	1.18±0.1	1.18±0.1	1.32±0.1	1.38±0.1	1.28±0.1	1.28±0.1	1.18±0.1	1.12±0.1
Serine	2.26±0.2	2.09±0.23	2.06±0.23	1.96±0.23	1.70±0.2	1.62±0.2	1.88±0.2	1.88±0.3	1.81±0.3	1.79±0.2	1.72±0.2	1.44±0.1
Glutamic	8.52±1.3	8.21±1.2	8.19±1.2	6.50±1.1	5.76±1.2	9.96±1.6	6.63±1.1	5.54±0.9	5.46±1	8.66±1.3	8.60±1.3	5.34±1
Proline	3.34±0.3	3.62±0.34	3.6±0.34	2.86±0.12	2.80±0.2	2.68±0.24	2.60±0.3	3.04±0.3	3.00±0.3	2.75±0.3	2.70±0.3	2.44±0.3
Glycine	0.92±0.1	0.79±0.11	0.77±0.11	0.80±0.1	0.86±0.09	0.92±0.11	0.80±0.1	0.72±0.08	0.71±0.08	0.85±0.1	0.82±0.1	0.74±0.08
Alanine	1.56±0.1	1.55±0.19	1.54±0.19	1.34±0.13	1.40±0.1	1.14±0.13	1.44±0.1	1.38±0.1	1.31±0.1	1.42±0.1	1.40±0.1	1.38±0.1
Cysteine	0.18±0.03	0.18±0.03	0.18±0.03	0.16±0.01	0.14±0.01	0.16±0.01	0.17±0.01	0.16±0.01	0.16±0.01	0.26±0.03	0.21±0.03	0.15±0.02
Valine	1.72±0.2	1.61±0.22	1.61±0.22	1.75±0.17	1.45±0.12	1.31±0.12	1.64±0.15	1.09±0.1	1.04±0.1	1.59±0.1	1.55±0.1	1.32±0.11
Methionine	0.98±0.1	0.95±0.14	0.95±0.14	0.97±0.11	0.81±0.07	0.95±0.1	0.77±0.1	0.85±0.07	0.84±0.07	1.11±0.15	1.01±0.15	0.85±0.1
Isoleucine	2.48±0.22	2.35±0.31	2.35±0.31	2.20±0.15	2.08±0.2	2.07±0.21	2.19±0.2	2.02±0.17	1.95±0.17	2.41±0.3	2.33±0.3	2.20±0.19
Leucine	3.02±0.3	2.45±0.3	2.45±0.3	2.63±0.23	2.40±0.16	2.22±0.23	2.48±0.22	2.18±0.15	2.11±0.15	2.73±0.3	2.66±0.3	2.35±0.18
Tyrosine	2.06±0.2	1.87±0.2	1.85±0.2	1.42±0.14	1.67±0.1	1.52±0.13	1.74±0.12	1.55±0.1	1.53±0.1	1.98±0.2	1.94±0.2	1.74±0.1
Phenylalanine	1.72±0.2	1.88±0.22	1.86±0.22	1.17±0.11	1.48±0.1	1.31±0.12	1.53±0.1	1.39±0.11	1.36±0.11	1.73±0.2	1.66±0.2	1.54±0.1
Histidine	1.80±0.2	1.94±0.23	1.91±0.23	1.55±0.25	1.83±0.2	1.47±0.11	1.68±0.12	1.57±0.11	1.56±0.11	1.82±0.2	1.79±0.2	1.60±0.1
Lysine	1.12±0.1	0.99±0.17	0.95±0.17	0.77±0.09	0.85±0.1	0.66±0.02	0.78±0.06	0.80±0.08	0.78±0.08	0.92±0.1	0.87±0.1	0.81±0.1
Tryptophan	0.94±0.1	0.98±0.16	0.95±0.16	0.83±0.1	0.79±0.08	0.73±0.11	0.79±0.07	0.85±0.1	0.82±0.1	0.99±0.11	0.95±0.11	0.91±0.1
Arginine	1.10±0.1	1.11±0.16	1.01±0.16	1.15±0.13	0.78±0.09	0.82±0.12	0.75±0.1	0.78±0.1	0.75±0.1	0.83±0.09	0.79±0.09	0.69±0.05
Total	35.10±3.3	33.93±2.9	33.61±2.9	29.26±2.7	27.98±2.6	30.08±1.9	29.19±2.5	27.18±2.9	25.74±3.0	33.12±3.1	32.3±3.1	26.62±2.8

Averaged data of analyzed Cheese samples in triplicates.

during storage period, while the number of *Enterobacteriaceae* remained high during the ripening/storage period, despite a slight decrease at the end of ripening period. All bacterial groups except for coliforms were maximum in young cheeses. Numbers of *Lactococcus* sp. were slightly higher than those of *Lactobacilli* and total mesophilic bacteria. The difference of *Lactococci* counts from the other groups was maximum three log units. The predominance of *Lactococcus* sp. during the early stages of raw milk cheeses ripening was reported (Manolopoulou et al., 2003). Lactic acid bacteria (*Lactococci, Lactobacilli* and *Enterococci*) were quantitatively the dominant groups, and change of their viable numbers was significant (P≤0:01) throughout the ripening period. Numbers of *Enterococcus* sp. in all samples of Ras cheese were almost the same in Blue cheese. The presence of *Enterococci* sp. in high numbers could be due to their tolerance to a wide range of environmental conditions such as low temperature, high salt content and acidity (Lorencová et al., 2012; Buňková et al., 2010). Because of these properties, although all microorganisms were effected from salt significantly (P≤0:05), *Enterococcus* sp. were not. *Enterococci* are a group of microorganisms that may influence the ripening process due to their proteolytic and lipolytic activities and their ability to stimulate acid production by some *Lactococci* (Sarantinopoulos et al., 2001). Total mesophilic aerobic bacteria increased reaching their highest numbers during a 45 day ripening period at cold storage, and then rapidly declined. Numbers of microorganisms indicative of the hygienic quality, such as coliforms, *Enterococcus* sp. and *Lactococcus* sp. were present in cheese at relatively high levels. These counts suggest that contamination was very high in raw milk. Numbers of coliforms and

Table 4. Means counts of microorganisms in Egyptian market cheeses.

Cheese type	Ripening/ storage period (month)	Aerobic mesophilic bacteria (10^6 cfu/g)	Coliform group (10^2 cfu/g)	Moulds and Yeasts (10^3 cfu/g)	Lactococci (10^4 cfu/g)	Enterococci (10^4 cfu/g)	Lactic acid bacteria (10^6 cfu/g)
Mish	6	7.59± 0.13^{b}	4.98± .15^{ef}	3.89 ± 0.21^{f}	7.13± 0.19^{a}	6.45± 0.22^{a}	10.24±0.09^{a}
	12	7.37±0.42b^{c}	5.07± .44de	4.05± 0.20^{ef}	6.94± 0.31^{b}	6.38± 0.87^{b}	9.66±0.12^{b}
	24	6.84± 0.06^{fg}	5.27± .59^{cde}	4.33± 0.63^{de}	6.75± 0.18^{c}	6.33± .44^{bc}	9.50±0.17^{bc}
	36	6.28± 0.04^{i}	5.34± .57^{cd}	4.74± 0.90^{c}	6.66± .06^{cde}	6.13± 0.54^{c}	9.23±0.08^{def}
	48	6.09± 0.18^{i}	5.53± .35^{bc}	4.82± 0.42^{c}	6.34± 0.37^{f}	5.88± 0.30^{d}	9.15±0.13^{ef}
Ras	6	7.13± 0.20^{de}	4.5± 0.23^{g}	4.05± 0.20^{ef}	6.67±0.20^{cd}	5.82±0.18^{de}	9.65±0.20^{b}
	9	6.95± 0.24^{ef}	4.63± 0.21^{g}	4.37± 0.43^{d}	6.58± .17de	5.78± .39de	9.55±0.13^{bc}
	12	6.65± 0.21^{gh}	4.72± .42^{gf}	4.79± 0.61^{c}	6.50± .13^{ef}	5.73± .47de	9.30±0.11^{de}
	24	6.51± 0.18^{h}	3.93± 0.23^{h}	5.01± 0.39^{bc}	6.40± 0.19^{f}	5.63± 0.38^{e}	9.13±0.07^{ef}
Blue-viened	6	7.92± 0.24^{a}	5.84± 0.25^{a}	4.82± 0.42^{c}	4.51± 0.14^{g}	5.88± 0.41^{d}	9.36±0.011^{cd}
	9	7.37± 0.21^{bc}	5.65± .26^{ab}	5.23± 0.16^{ab}	4.38± 0.21^{g}	5.78± .44de	9.30±0.06^{de}
	12	7.22± 0.58^{ed}	5.27± .19^{cde}	5.37± 0.39^{a}	4.00± 0.09^{h}	5.73± .30de	9.08±0.08^{f}

Microbiological composition of Egyptian market cheeses (means ± SD). Means log counts in triplicates.

Enterococcus sp. were not reduced significantly (P≤0:05), while numbers of *Lactobacilli* sp. were also reduced significantly depending on the ripening time (P≤0:01), but they remained alive. This can be explained by the pH levels and the quantity of lactic acid. Counts of yeasts and moulds were in Mish cheese similar to other findings in Ras cheeses. During the ripening/storage period, the numbers were not significantly decreased (P≤0:01), and they had relatively high counts in Blue cheeses. Yeasts were present at various levels among the distinct cheeses, grouped from dairy markets, the differences in numbers may be due to the distinct pH and salt concentrations found between the corresponding cheeses, although no significant correlations resulted. Occurrence of yeasts in cheeses was variable, because they have been associated with the production of flavour compounds as a result of their relatively strong proteolytic and lipolytic activities. However, scant information is available regarding the contribution of yeasts to synthesis of BAs in foods: a histidine-decarboxylase activity was found in yeasts of the genera Debaromyces and Candida isolated from fermented meat (Montel et al., 1999) and such an activity was actually above that observed in lactic acid bacteria. Macedo et al. (1995) found that the presence of yeasts was closely related to lactic acid utilization, while their contribution to the ripening process was due to their proteolytic and lipolytic activities. In this study, the number of microorganisms such as yeasts, moulds and coliforms causing spoilage of cheeses by their putrefactive effects were decreased slightly. Formation of basic compounds from proteolysis could be as a resulting of changing of pH and a decrease of acidity. As recommendation, the permissible level of biogenic amines stipulated by Egyptian Organization for Standardization and Quality Control (EOS, 1996) should be modified to meet the more safe standard adopted by Food Drug Administration (FDA, 2001) and their levels can be lowered by using good quality raw milk and maintaining hygiene standards during manufacturing and storage processes.

Conclusions and recommendations

The main feature influencing the BAs formation is the extent of growth of microorganisms, like *Enterococci* sp. characterized by decarboxylase activity. The presence of high contents of BAs in Mish and Ras cheeses could be related to the enzymatic activity of proteases derived from microorganisms, or from another factor, that is important from a qualitative point of view, that is, in relation to the type of amino acids provided to the amino acid decarboxylating microbiota, in particular tyrosine. Therefore, a large amount of BAs in cheese reflects the bad hygienic conditions under which they are produced and stored. Accordingly, the levels of biogenic amines in different cheeses should be in accordance with the safe permissible limit recommended by FDA to ensure human safety.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Assessment of bacterial and fungal spoilage of some Nigerian fermented and unfermented foods

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The study was aimed at evaluating the microbial spoilage of selected Nigerian fermented and unfermented foods. A total of four fermented and unfermented food samples were used for this investigation. Microbial and sensory evaluations of the food products during storage were carried out using standard procedures. During storage, the bacteria counts were observed to range from 2.2 × 10^5 to 4.8 × 10^5 CFU/mL and from 2.5 × 10^3 to 5.0 × 10^4 CFU/mL, for the unfermented and fermented food products, respectively. Similarly, the fungal counts ranged from 1.8 × 10^3 to 2.9 × 10^3 CFU/mL and from 0 to 5.70 × 10^3 CFU/mL for the unfermented and fermented food samples, respectively. *Klebsiella aerogenes*, *Lactobacillus plantarum*, *Leuconostoc* sp., *Micrococcus varians*, *Proteus mirabilis*, *Streptococcus faecalis, Staphylococcus epidermidis*, *Aspergillus niger*, *Aspergillus flavus*, *Cladosporium herbarum*, *Geotrichum candidum*, *Mucor mucedo*, *Neurospora sitophilia* and *Penicillium* sp. bacteria and fungi that were recovered from the food samples during storage. The sensory evaluation of the food products showed the fermented ones being more acceptable to panelists than the unfermented ones. This could indicate that the palatability of the fermented food samples only experienced slight or no changes during storage, when compared to the unfermented ones.

Key words: Fermented food, unfermented food, microbial spoilage.

INTRODUCTION

It is reported that fermented foods constitute about 25% of the foods consumed worldwide. Fermented foods have many advantageous attributes, which include improved nutritional value and safety against pathogens over non-fermented foods. Generally, these foods are normally considered to be safe against foodborne diseases because of the advantage of low pH, which is due to the presence of organic acids produced during fermentation. In Africa, because a majority of the fermented foods are produced at household levels, the issue of hygiene is a major concern (All and Dardir, 2009; Gadaga et al., 2004).

Both fermented and unfermented food products are known to be susceptible to spoilage during storage. Their spoilage during storage is attributed to the presence of microorganisms and extracellular enzymes produced, which breakdown the food product into new substances resulting into changes in their organoleptic properties

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(Fadahunsi et al., 2013). Chance inoculation, activities of spoilage organisms and the humid condition in the Tropics are some of the factors responsible for spoilage of these foods during storage. While starter cultures have been used to ensure uniformity in the composition of some fermented foods, their reduced shelf life still remains a major problem worldwide. The ingestion of products contaminated with these microorganisms could be a potential health threat hence there is the need to control microbial contamination of fermented foods and feeds.

Filamentous moulds and yeast are common spoilage organisms of food products as fermented milk products, cheese, bread, stored crops and feed such as hay and silage (Filtenborg et al., 1996). *Penicillia* and *Aspergilli* species have been reported as spoilage organisms during storage of a wide range of foods where they may produce a number of mycotoxins (Samson et al., 2002). Several authors (Pitt and Hocking, 1999; Magnusson and Schnurer, 2001) have also implicated yeasts such as *Candida parapsilosis, Rhodotorula mucilaginosa, Kluyveromyces marxians and Derbaromyces hansenii* as common spoilage organisms of yoghurt and other fermented food products.

Apart from food spoilage, health hazards from fungal mycotoxins had been well documented. For instance, the large consumption of grains infected with sclerotia of *Claviceps pupurea* caused several thousand deaths in the 10^{th} and 11^{th} century in central Europe (Pohland, 1993). It is estimated that between 5 and 10% of the world's food products is lost due to fungal deterioration (Pitt and Hocking, 1999). The growth of these undesirable fungal species in foods has been a worldwide concern over the years because of its effects on man's health and consequent economic loss due to food storage. During food spoilage, which is a metabolic process, the spoilt food becomes undesirable and unacceptable for human consumption due to changes in sensory characteristics.

Fermented foods such as gari, ogi, fufu and mawe are found all over the world. Fermentation has offered a means of food security in Nigeria; where over 52% of respondents in a previous study in 2003 indicated that about 25% of their monthly income is spent on fermented food products (Aderiye and Laleye, 2003). This paper therefore examines and discusses the desirability of consuming part of spoiled fermented food products as reported by Aderiye and Laleye (2003). It is also aimed at evaluating the types and extent of microbial spoilage of some selected Nigerian fermented and unfermented foods.

MATERIALS AND METHODS

A total of eight different food samples, divided into unfermented (pounded yam, yam, rice and beans) and fermented ('Eba', 'Eko', 'Fufu' and 'Kati') food products were used in this study. The raw food products were purchased from the local market in Ado-Ekiti, Ekiti State in Nigeria. To ensure uniformity and prevent any pre-storage contamination, the purchased raw food products were prepared locally in the laboratory using aseptic techniques. Each of the prepared food samples was transferred into and wrapped with sterile polythene bags (*ca* 0.2 mm thick), while some were left unwrapped. Food samples from 'Eko' and 'Kati' were wrapped in the leaves of *Thaumatoccus daniella*. This was to ensure that the samples were in the same state as they were normally packaged in the locality. Detail description of the food products and their preparation have been described elsewhere (Aderiye and Lalaye, 2003). All the food products were stored under hygienic conditions at refrigeration and ambient temperatures.

Microbiological analyses were carried out using conventional microbiological procedures. The total microbial count on the food sample was determined by the pour plate method using standard microbiological techniques. Nutrient agar and Malt extract agar were used to culture bacteria and fungi, respectively. All bacterial and fungal counts were expressed as colony forming units per milliliter (CFU/mL) and propagules and cells/mL respectively. In all cases, each analysis was carried out in triplicates. The isolation and identification of the bacteria and fungi were carried out as described elsewhere **(**Barnett and Hunter, 1992; Burgess and Sum, 1994; Holt et al., 1994). The occurrence of each isolate in the food samples was determined as a percentage of the total number of food samples and recorded as the frequency of occurrence. The frequency of occurrence of isolates in the food samples was calculated using the following formula:

$$\%\ \text{frequency of occuerence} = \frac{\text{No of microbial occurrence in the food samples}}{\text{Total number of food samples}}$$

Sensory properties such as appearance, colour, odour, taste, texture and general acceptability were also used in an 11-point 'Hedonic' scale to test the acceptability and rejection of the food samples. The detection of spoilage was done through physical examination of the stored food samples. The wrapped food samples were observed daily for changes in appearance, texture, aroma and taste. The day of onset of each defect or sign of spoilage and the types were recorded.

Each of the experiments was carried out in triplicates and conducted twice. All the statistical analyses were carried out using the SPSS computer statistical software. The comparison of means was done using the One-Way Analysis of Variance Test.

RESULTS

The microbial quality of the food samples is shown in Figures 1 and 2. When the food samples were examined within one hour of processing, no microbial contamination was recorded. As shown in Figure 1, in all the food samples, bacterial colonization was observed within 24 h after production. The bacterial counts in the food samples were observed to increase with increase in storage time. At the end of 96 h storage period, the bacterial counts from the unfermented food samples ranged from 2.2×10^5 to 4.8×10^5 CFU/mL, for yam and pounded yam, respectively. In the fermented food samples, the bacterial count was observed to range from 2.5×10^3 to 5.0×10^4 CFU/mL, for 'Kati' and 'Eba', respectively (Figure 1). In all cases, the bacterial counts in the unfermented food samples were significantly higher than those in the fermented food samples ($P \leq 0.05$). Even after 96 h storage, 'Eba' still

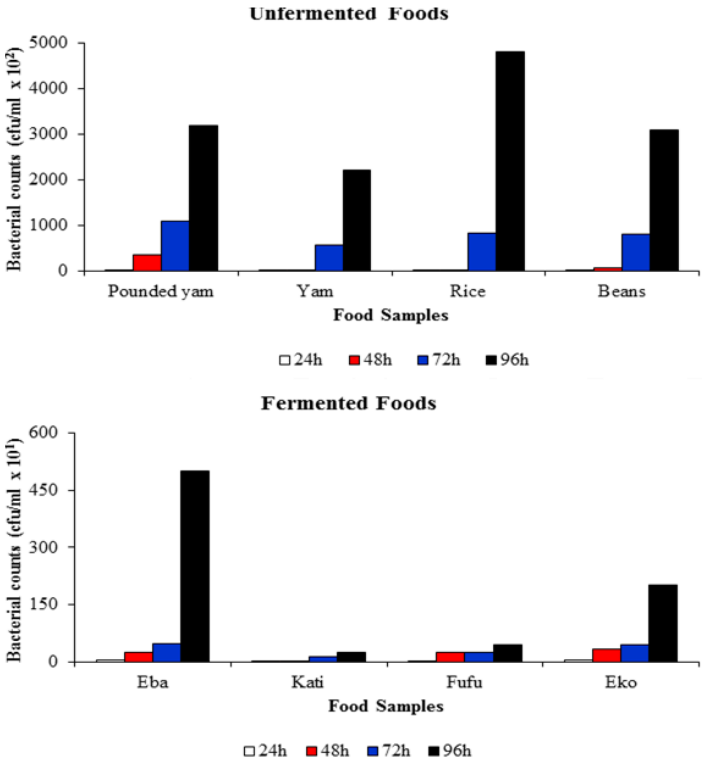

Figure 1. Average bacterial counts of the food samples during the period of storage.

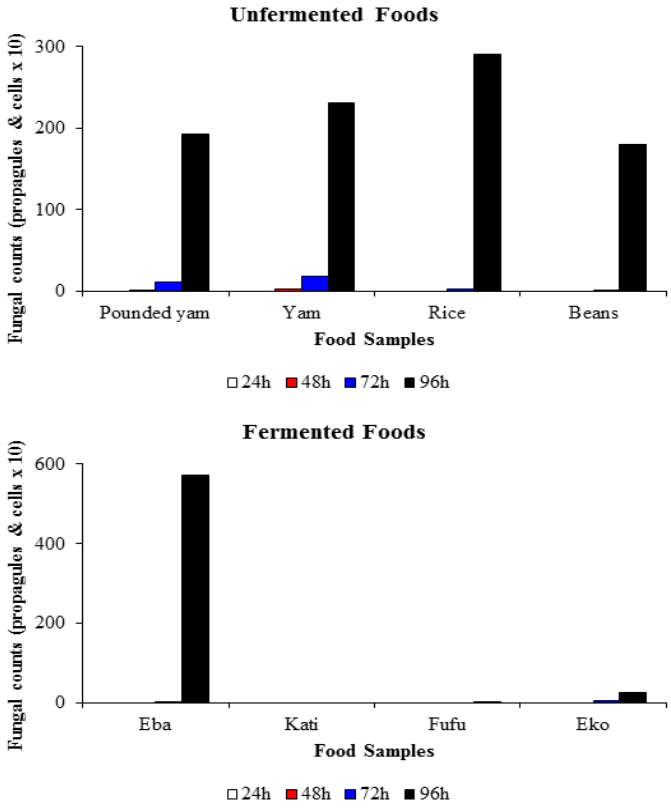

Figure 2. Average fungal counts of the food samples during the period of storage.

Table 1. Occurrence and distribution of bacteria in the food samples.

Food sample	*Kleb.*	*Lact.*	*Leuc.*	*Micr.*	*Prot.*	*Strep.*	*Staph.*
Pounded yam	+	-	-	+	+	+	+
White rice	+	-	-	+	+	-	+
Beans	-	-	-	-	+	-	+
Yam	-	-	-	-	-	+	+
Eba	-	-	-	-	+	+	+
Kati	-	+	+	-	-	-	+
Fufu	-	+	-	-	-	-	+
Eko	-	-	-	+	-	-	+

'+' and '-' mean present and absent respectively. *Kleb.= Klebsiella aerogenes, Lact.= Lactobacillus plantarum, Leuc.= Leuconostoc* sp., *Micr.= Micrococcus varians, Prot.= Proteus mirabilis, Strep.= Streptococcus faecali, Staph.= Staphyloccus epidermidis.*

Table 2. Occurrence and distribution of fungi in the food samples.

Food sample	*A. fla*	*A. nig*	*Clad*	*Geot*	*Muco*	*Neur*	*Peni*
Pounded yam	-	-	-	-	-	+	+
White rice	-	-	-	+	-	+	-
Beans	+	-	-	+	-	-	-
Yam	-	+	+	-	-	-	-
Eba	-	+	-	-	+	-	+
Kati	-	-	-	-	-	-	-
Fufu	+	-	-	-	-	-	-
Eko	+	-	-	+	-	+	+

'+' and '-' mean present and absent, respectively. *A. fla= Aspergillus flavus, A. nig= Aspergillus niger, Clad= Cladosporium herbarum, Geot= Geotrichum candidum, Muco= Mucor mucedo, Neur= Neurospora sitophilia, Peni= Penicillium* sp.

had a load (5×10^4 CFU/mL) far lower than the pounded yam stored for 24 h (2.2×10^5 CFU/mL).

As shown in Figure 2, fungi colonization in the unfermented and fermented food samples was observed to commence within 48 and 72 h, respectively. At the end of 96 h storage, the fungal counts were observed to range from 1.8×10^3 propagules/ cells and 2.9×10^3 propagules /cells in the unfermented food samples. In the fermented food samples, fungal count was observed to range from 0 to 5.70×10^3 propagules/cells, respectively. In all the fermented foods, there were no traces of fungal growth for 48 h. Throughout the 96 h storage period, no fungi colonization was observed in 'kati' (Figure 2). As was observed with the bacterial counts, the fungal counts in the unfermented food samples were significantly higher than counts in the fermented food samples ($P \leq 0.05$).

During the storage period, a total of eight bacterial strains were isolated from the food samples. The isolated bacterial strains include *Klebsiella aerogenes*, *Lactobacillus plantarum*, *Leuconostoc* sp., *Micrococcus varians*, *Proteus mirabilis*, *Streptococcus faecalis* and *Staphylococcus epidermidis* (Table 1). In the case of fungi, eight strains were isolated from the food samples. The strains were *Aspergillus niger*, *Aspergillus flavus*, *Cladosporium herbarum*, *Geotrichum candidum*, *Mucor mucedo*, *Neurospora sitophilia* and *Penicillium* sp. (Table 2).

With respect to the frequency of occurrence of microbial isolates from the food samples, a rank of the bacteria revealed that *Leuconostoc* sp (13%) and *Staphylococcus epidermidis* (45%) were the least and the most occurring bacteria in the food samples, respectively. In the case of fungi, *Aspergillus niger* and *Penicillium* sp. were observed to be the least (40%) and the most occurring fungi in the food samples, respectively (Figure 3).

With respect to the organoleptic attributes of the food samples during storage, although there was a significant difference between the different food samples, a general trend was that the fermented food samples were more acceptable to the panellists than the unfermented food samples. Only a few number of respondents accepted

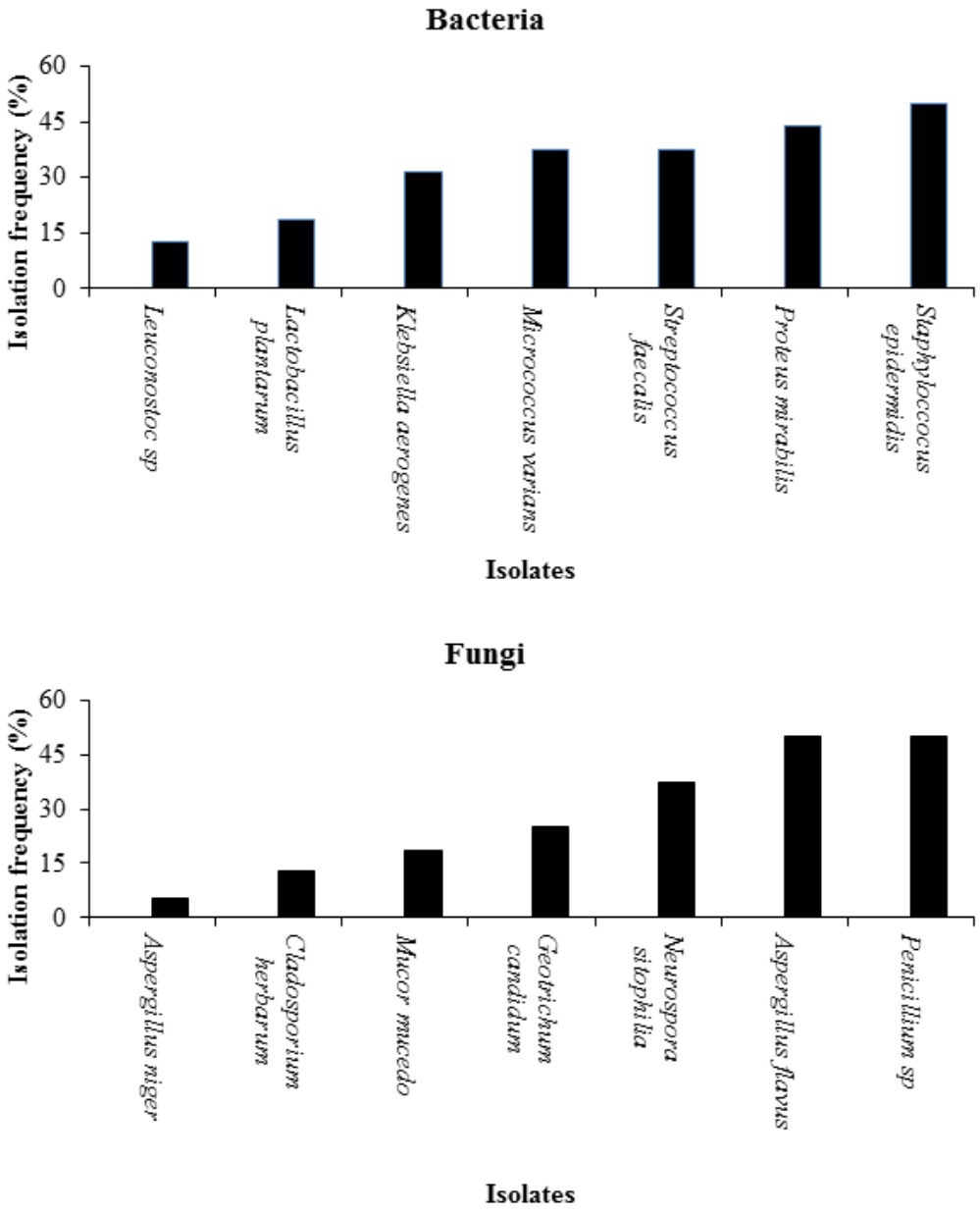

Figure 3. Rank of the frequency of occurrence of the microbial isolates in the food samples.

beans stored for 96 h while 'Kati' was the most acceptable of the food samples. A daily decrease in the means of the attribute was observed in all the unfermented food samples while the arithmetic difference in the means of the fermented food samples was observed to be negligible (Table 3).

A comparison of storage at 25 and 4°C revealed that off-colour, mouldiness and objectionable taste were the commonest indicators of spoilage in all the food samples. Stringiness and sliminess were observed to be limited to the unfermented food samples. The results revealed that all the food samples were affected by one or more fungal species as shown by mouldiness (Table 4). The foods stored at refrigeration temperature had longer acceptable days (5 to 76 days) and a longer shelf life (4 to 56 days) than those stored at room temperature, which were 2 to 22 days and 2 to 17 days for acceptable days and longer shelf life, respectively).

DISCUSSION

A general observation in this study was the presence of higher microbial load during storage in the unfermented food samples than in the fermented. The higher storability and lower spoilage rate of the fermented food samples are therefore not surprising as fermented foods had been

Table 3. General Sensory evaluation of the stored food samples.

Food sample	Fresh	24 h	48 h	72 h	96 h
Pounded yam	9.8[a]	5.6[c]	4.7[c]	2.6[d]	1.5[d]
Yam	9.6[a]	6.0[c]	5.4[b]	4.6[b]	2.4[c]
Rice	10.0[a]	6.5[b]	5.1[c]	3.5[c]	1.98[c]
Beans	9.6[a]	6.1[c]	4.8[c]	2.5[d]	1.2[d]
Eba	9.9[a]	8.6[a]	8.5[a]	8.0[a]	7.0[b]
Kati	9.9[a]	8.5[a]	8.5[a]	8.2[a]	8.0[a]
Fufu	9.8[a]	8.3[a]	8.0[a]	7.7[a]	7.5[b]
Eko	10.0[a]	8.2[a]	8.2[a]	7.8[a]	7.5[a]

Values in the same column not followed by the same superscript are significantly different ($p \leq 0.05$).

Table 4. Observed days of spoilage symptoms of the food samples stored at 25 and 4°C.

Parameter	Pounded yam	Yam	Rice	Beans	*'Eba'*	*'Kati'*	*'Fufu'*	*'Eko'*
Appearance								
Off-colour	3 (4)	2 (3)	2 (4)	4 (6)	5 (17)	17 (56)	9 (35)	5 (15)
Mouldiness	4 (7)	3 (6)	5 (8)	5 (9)	7 (20)	20 (64)	12 50)	8 (21)
Texture								
Stringiness	3 (-)	2 (-)	2 (5)	- (-)	- (-)	- (-)	- (-)	- (-)
Sliminess	- (-)	1 (4)	2 (4)	1 (4)	- (-)	- (-)	- (-)	5 (-)
Watery	3 (-)	- (-)	2 (5)	3 (-)	- (-)	- (-)	- (-)	7 (-)
Softness	2 (-)	3 (-)	2 (4)	- (-)	6 (20)	- (-)	9 (-)	6 (-)
Separated	2 (3)	- (-)	- (-)	- (-)	- (-)	- (-)	- (-)	7 (18)
Aroma								
Off-odour	2 (4)	2 (4)	2 (4)	2 (5)	7(22)	19 (74)	12(50)	8 (17)
Putrid	- (-)	- (-)	2 (3)	2 (5)	- (-)	- (-)	- (-)	- (-)
Taste								
Objectionable	3 (4)	2 (4)	1 (5)	2 (6)	5 (21)	22 (72)	10(62)	9 (19)
Sourness	2 (3)	- (-)	- (-)	- (-)	- (-)	18 (70)	- (-)	9 (5)
Rejected	2 (5)	2 (5)	3 (7)	2 (7)	8 (22)	22 (76)	15(52)	11(24)

Values in parenthesis represent storage at 4°C while all other values indicate at 25°C. '-' indicate not detected. *'Kati' and 'Eko'* were wrapped in leaves during the storage period.

reported to have a higher shelf life (Steinkraus, 1997; Ogunbanwo et al., 2004). This could be attributed to the release of antimicrobial metabolites by the fermenting organisms into these food substrates (Adebayo and Aderiye, 2010). The antimicrobial compounds include bacteriocin, diacetyl, hydrogen-peroxide and organic acid (Adebayo and Aderiye, 2007).

The differences in the microbial contamination of pounded yam and yam both from the same raw material could be attributed to the higher level of moisture content in the pounded yam. Frazier and Westhoff (2007) had reported that high water activity favoured the growth of contaminating microorganisms. The higher storability of the refrigerated food samples (4°C) could be attributed to the fact that the spoilage organisms are mesophiles hence the low temperature (4°C) inhibited their metabolic activities and thus led to limited growth, inhibition or death in causes of some organisms (Bracket, 1997). Also, the highest shelf life (76 days) recorded by 'Kati' may be due to its low pH, low moisture and high fibre contents. Such food tends to be more stable microbiologically (Frazier and Westhoff, 2007). One of the findings of this study, that mouldiness is a perceived indicator of deterioration in the selected Nigerian foods, predicates

the need to evolve means to prevent fungal spoilage of fermented Nigerian foods.

A total of seven fungal strains belonging to six genera were found to be responsible for the spoilage of the food samples used in this study. The most predominant genera were *Aspergillus* and *Penicillium* which constituted more than 66% of the total fungal isolates. Previous workers have reported the predominance of these organisms in other food systems like mawe (Hounhouighan et al., 1993); 'burukutu' (Sanni et al., 1999) and dried sausages (Mataragas et al., 2002).

The prevalence of *Aspergillus* and *Penicillium* species could be due to their sporulating ability; hence they easily contaminate the environment (Frisvad and Samson, 2007). Food samples from 'Eko' recorded the highest number of fungal contamination (66.66%). This could be attributed to its having the highest moisture content (55.04%). Foods with high moisture content generally encourage fungal growth because spore germination and hyphal growth require moisture (Odigie, 2000). Another reason could be due to the wrapping of 'Eko' with leaves of *Thaumatococcus daniella*, which could be a potential source of contamination. According to Fadahunsi and coworkers (2013), the occurrence of the isolates could be attributed to the biodegrading potential of the microflora to convert diverse substrates by secretion of extracellular enzymes.

In the present study, the spoilage fungi isolated from the food samples are common microorganisms associated with the environment. Contamination of the food substrates by these fungi was expected as the ambient temperature of storage and pH (4.1-4.6) of the fermented food samples favour fungal proliferation (Brock and Madigan, 2003). Many of the isolated fungi are of clinical importance because of their potentials in causing human diseases. For instance, strains of *Aspergillus* and *Penicillium* spp. are known to produce mycotoxins which can result in mycotoxicoses (Smith et al. 1995). The toxins can damage liver, causing cirrhosis and can also induce tumor. Hence the assumption of some consumer of the fermented foods in southwest Nigeria that these mould - infested foods are only unaesthetic but not dangerous to health should be discouraged, as such foods could be potential sources of mycotoxicosis.

The 'Eko' had the highest occurrence and distribution of genera of fungal contaminants (5) while 'Fufu' had the least (2). The differences in the occurrence of these fungi in the food samples may be attributed to the differences in their nutrient status. For instance, Adebayo and Aderiye (2009) reported that 'Eko' had higher metabolisable sugar (1.63%) and moisture (55.04%) content, than 'Fufu' (0.048 and 42.15%) thus predisposing the former to a higher level of fungal attack. The delay in the incidence of the spoilage organisms until after 72 h could be attributed to inadequate moisture level required for spore germination prior to 72 h of storage or to the antimicrobial effect of some compounds liberated by LAB into the food substrates during fermentation (Ryhz et al., 2008; Adebayo and Aderiye, 2010).

Foods generally become more susceptible to pathogens as storage progressed due to the absorption of moisture from air and thus make the tissues more accessible to hydrolytic enzymes of the attacking pathogens. It is indicated that although microbial fermentation has played a vital role in food processing in Nigeria, because many of the indigenous fermented foods are processed through spontaneous and uncontrolled natural fermentation, despite its advantages, the accompanying microflora are indicated to be causes of spoilage and in some case toxicity (Aderiye and Adebayo, 1999).

Infestation by spoilage fungi caused some physical defects like off-colour, loss of firmness, loss of aroma, and softness on the food samples. Frazier and Westhoff (2007) attributed the change in texture to the activity of the spoilage organisms that encouraged the extraction of mineral nutrient from the food substrates. Loss of aroma was however attributed to the fermentation of the non-proteinaceous constituents of the food substrates. Fungal activity has been reported to cause a rapid deterioration of quality of food substrates (Teniola and Odunfa, 2002; Samson, 2007; Adebayo and Aderiye, 2009).

Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

This study which was aimed at evaluating the microbial spoilage of selected fermented and unfermented foods in Nigeria have revealed that during storage, both fermented and unfermented foods are susceptible to spoilage, although the onset of spoilage is faster in the unfermented one. A variety of bacteria and fungi species were also observed to be responsible for spoilage of the food samples during storage. With storage at room temperature, the onset of spoilage was remarkably faster in the fermented food samples than the fermented ones. In all, a sensory evaluation showed that the fermented foods were more acceptable to panelists than the unfermented ones after storage. This could indicate that the palatability of the fermented food samples only experienced slight or no changes during storage, when compared to the unfermented ones.

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Full Length Research Paper

Antioxidant peptides from freshwater clam extract using enzymatic hydrolysis

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The enzymatic hydrolysate of freshwater clam (*Corbicula fluminea*) extract was prepared using commercially available proteases. The antioxidant activity of the hydrolysate was evaluated by reducing power and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid free radical decolorization assays. The hydrolysate, especially its fraction purified by gel filtration, had a good antioxidant activity. The Trolox equivalent antioxidant capacity of the fraction with the highest radical scavenging ability reached 158.04 ± 2.43 µg/mg, which was sevenfold higher than that of the original hydrolysate. Molecular weight distribution analysis revealed that peptides over 1000 Da in the hydrolysate had better abilities to donate electrons, whereas peptides below 1000 Da more effectively eliminated radicals. Therefore, peptides from freshwater clam extract could be employed as potential natural antioxidants, performing their activities via different mechanisms.

Key words: Antioxidant peptides, freshwater clam extract, enzymatic hydrolysate, molecular weight distribution.

INTRODUCTION

Freshwater clam (*Corbicula fluminea*) is a widely consumed bivalve in Asia. In addition to traditional fresh or dry products, freshwater clam essences (hot water extracts) are currently being marketed as a nutritional supplement in Taiwan. Various physiological functions of freshwater clam have been recognized. Freshwater clam extracts have shown a hypocholesterolemic effect for accelerating cholesterol degradation as well as excreting neutral sterols and bile acids (Chijimatsu et al., 2008). The muscle protein hydrolysates of freshwater clam have demonstrated a high inhibitory effect on angiotensin I-converting enzyme *in vitro* and antihypertensive activity by oral administration *in vivo* (Tsai et al., 2006). Hence, more attention is being paid to the recovery of such bioactive substances from freshwater clam (Chijimatsu et al., 2009; Lin et al., 2010; Sun et al., 2011).

Many diseases such as diabetes, atherosclerosis, neurodegenerative disorders, etc. have been confirmed to be related with oxidative stress. Therefore, antioxidants are widely used as ingredients in dietary supplements to maintain good health (Elias et al., 2008). The use of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene is currently restricted to food because they can induce DNA damage and toxicity. Consequently, numerous efforts have been exerted on the search for safe and natural antioxidants from animal and plant sources. In the past few years, many peptides that possess antioxidant activities against reactive oxygen species and free radicals have been identified. Such peptides include those from milk (Pihlanto, 2006), porcine myofibrillar protein (Saiga et al., 2003), egg yolk protein (Sakanaka and Tachibana, 2006), canola (Cumby et al., 2008), and chickpea protein (Li et al., 2008). Peptides originating from various fish and shellfish have also exhibited good antioxidant activities in different oxidative systems (Je et al., 2005; Mendis et al., 2005; Kim et al., 2007). Jellyfish collagen peptides ranging from 400 to 1200 Da have displayed high superoxide

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anion-scavenging (IC_{50} = 21.9 μg/mL) and hydroxyl radical-scavenging (IC_{50} = 16.7 μg/mL) activities in vitro (Zhuang et al., 2009). The heptapeptide His-Phe-Gly-Asp-Pro-Phe-His (MW = 962 Da), derived from fermented marine blue mussel (*Mytilus edulis*) could effectively scavenge superoxide, hydroxyl, carbon-centered, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. The related IC_{50} values are 21, 34, 52, and 96 μM, respectively (Rajiapakse et al., 2005). However, studies on antioxidant peptides from freshwater clam are still limited.

In the current study, to evaluate the antioxidant activeties of the peptides from freshwater clam, the enzymatic hydrolysate was prepared from the hot water extract of freshwater clam, and the corresponding antioxidant activities were evaluated by reducing power and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical decolorization assays. The antioxidant peptides were purified by gel filtration and reverse phase (RP) high-performance liquid chromatography (HPLC). The composition of the peptides was determined using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

MATERIALS AND METHODS

The freshwater clams (*C. fluminea*) were purchased from a local fish market, transported on ice to the laboratory, and frozen at -20 °C until analysis. Crude protease G (from *Aspergillus oryzae*), peptidase R (from *Rhizopus oryzae*), and peptidase G (from *Aspergillus oryzae*) were supplied by Amano Enzyme Company (Naoya, Japan).

Folin–Ciocalteu's phenol reagent, bovine serum albumin (BSA), Leu-Gly, β-mercaptoethanol, *o*-phthaldialdehyde (OPA), trichloroacetic acid, potassium ferricyanide ($K_3Fe(CN)_6$), ferric chloride ($FeCl_3$), trifluoroacetic acid (TFA), ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analog), and Sephadex G-25 were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Merck Ltd. (Darmstadt, Germany).

Preparation of the hydrolysate

Whole freshwater clams (1000 g) were added to tap water in a 1:1 (w/w) ratio and boiled for 40 min according to a commercial procedure. The resulting liquid was filtered and concentrated to 400 mL. After the pH adjusted to 6.0 using NaOH, the hydrolysate was directly prepared from this condensed hot water extract. Protease G, peptidase R, and peptidase G were added, with the ratio of each enzyme to the initial freshwater clams as 1:250 (w/w). The reaction mixture was then incubated at 45°C for 24 h and subsequently heated at 90°C for 10 min to inactivate the enzymes. The supernatant obtained by centrifugation at 20 000×*g* for 20 min was lyophilized as the hydrolysate.

Measurement of soluble protein content

The soluble protein content was measured by the Lowry method (Lowry et al., 1951) using BSA as a standard.

Measurement of peptide content

The peptide content was measured by OPA using Leu-Gly as a standard according to the method of Church et al. (1983). Prior to the measurement, the sample solution (50 mg/mL) was pumped through a 0.22 μm membrane and an ultrafiltration membrane with a molecular weight cutoff of 5000 (Millipore, Bedford, MA, USA).

Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay was performed according to the method described by Re et al. (1999). The previously prepared $ABTS^{+\bullet}$ solution was diluted to an absorbance of 0.7 ± 0.02 at 734 nm using sodium phosphate buffer (20 mM, pH 7.8). After the sample (300 μL) was added to 2.7 mL of dilute $ABTS^{+\bullet}$ solution, the resultant mixture was vigorously shaken and allowed to stand in the dark for 6 min at room temperature. The absorbance at 734 nm was recorded as A_{sample}. The blank and the control were prepared in the same way as the sample, using ddH_2O and corresponding solvents instead of the sample and the test reagents, respectively. The percentage of $ABTS^{+\bullet}$ radical scavenging activity was calculated as follows:

$$\text{ABTS}^{+\bullet}\text{radical scavenging activity (\%)} = \frac{A_{blank} - \left(A_{sample} - A_{control}\right)}{A_{blank}} \times 100\%$$

A standard curve of Trolox ranging from 0 μg/mL to 6 μg/mL was also prepared in the same manner. The final $ABTS^{+\bullet}$ radical scavenging activity of the samples was expressed as microgram Trolox equivalents per milligram.

Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). The sample (1 mL) was mixed with 1 mL of 20 mM sodium phosphate buffer (pH 7.0) and 1 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated in a water bath at 50°C for 20 min, and trichloroacetic acid (10%, 0.5 mL) was added. After centrifugation at 750 × *g* for 10 min at room temperature, 2 mL of supernatant was collected, to which 2 mL of deionized water and 400 μL of 0.1% $FeCl_3$ were added. The absorbance of the reaction mixture at 700 nm was measured and recorded as A_{sample}. The reducing power (*A*) was calculated from the increase in the absorbance at 700 nm, as follows:

$$A = A_{sample} - A_{blank} - A_{control}$$

Purification of antioxidant peptides in the hydrolysate

The peptides in the hydrolysate were purified by gel filtration chromatography using a fast protein liquid chromatography AKTA explorer 10S (GE Healthcare, Uppsala, Sweden). After filtration through a 0.22 μm membrane, the hydrolysate (10 mg/mL, 200 μL) was loaded on a Sephadex G-25 column (16 × 25 mm) and eluted by deionized water at a flow rate of 1 mL/min. Each 1.0 mL fraction was collected until no protein was monitored by the absorbance at 280 nm.

The fraction obtained from the gel fraction, which possessed the highest $ABTS^{+\bullet}$ radical scavenging activity, was further separated using an RP-HPLC system (Jasco intelligent HPLC model 2080, Inc., Jasco, Tokyo, Japan) based on the absorbance at 220 nm. The lyophilized hydrolysate fraction (20 mg) was dissolved in 2 mL of 0.1% TFA. About 20 μL of this sample was injected into a TSK-gel ODS-80Ts column (5 μm, 4.6 mm × 250 mm, TosohBioscience

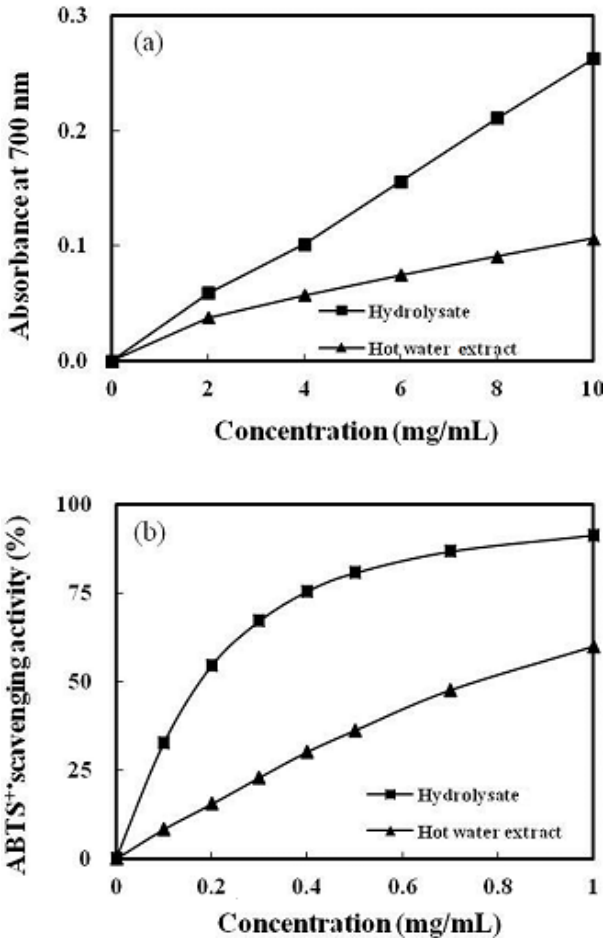

Figure 1. Reducing power and $ABTS^{+\bullet}$ radical scavenging activity of the hydrolysate from freshwater clam extract at various concentrations (a) Reducing power; (b) $ABTS^{+\bullet}$radical scavenging activity.

LLC, Tokyo, Japan) and eluted using a two-solvent system [solvent A: 0.1% (v/v) TFA in deionized water; solvent B: 0.1% (v/v) TFA in 80% acetonitrile] at a flow rate of 0.8 mL/min. The gradient elution was as follows: 98% A in 0 min to 5 min, 98% to 50% A in 5 min to 55 min, 50% to 5% A in 55 to 70 min, and 5 to 100% A in 70 to 90 min.

Molecular mass analysis

Molecular mass analysis was performed using a MALDI-TOF-MS system (AutoFlex, Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in the reflector and positive ion modes. External mass calibration was performed using a standard peptide mixture (Bruker Daltonics). The sample was diluted with 0.1% TFA aqueous solution, and α-cyano-4 hydroxycinnamic acid (CCA) was used as the matrix.

Statistical analysis

All analyses were carried out in triplicate, and results are reported as the mean ± standard deviation (SD). Significant differences were analyzed by two-way ANOVA using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). Differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Enzymatic hydrolysate of freshwater clam extract

The recovery of proteins in the hot water extract and enzymatic hydrolysate were calculated as 2.22 ± 0.06% and 2.13 ± 0.12%, respectively. The peptide contents were also determined as 0.12 ± 0.003 and 0.46 ± 0.01 mM/(mg·mL). The low recovery of proteins from raw freshwater clam was found not only in the current study, but also in other previous ones. Chijimatsu et al. (2011) have obtained approximately 1.5% (w/w) proteins from raw freshwater clam by hot water extraction. Tsai et al. (2006) have also pointed out that residual meat after hot water extraction still accounts for over 80% of the total weight of freshwater clam meat. In the present study, the recovery of proteins had a weak loss after hydrolysis. However, the peptide content remarkably improved for a wide variety of smaller peptides, and free amino acids were generated by enzyme effects.

Antioxidant activity of the hydrolysate

Antioxidants play roles in multiple reaction mechanisms. An efficient way of assessing antioxidants is to take into account their oxidation-reduction potentials, i.e., measuring their reduction powers. Another generally applicable method is to examine their free radical scavenging activities (Jiménez-Escrig et al., 2001). In the current study, the antioxidant activity of the hot water extract and its hydrolysate was evaluated via reducing power and TEAC assays with the $ABTS^{+\bullet}$. radical. As shown in Figure 1a, the reducing power of the extracts with and without subsequent hydrolysis both increased within the concentration range of 0 to 10 mg/mL. The reducing power of the hydrolysate was much stronger than that of the hot water extract (named as nonhydrolysate). The TEAC assay results (Figure 1b) were similar. The $ABTS^{+\bullet}$ radical scavenging activity of the two extracts increased with their concentration (0 mg/mL to 1 mg/mL), and the increasing trend of radical scavenging effect was more noticeable in the enzymatic hydrolysate. Using the standard curve of Trolox, the TEAC values of the nonhydrolysate and enzymatic hydrolysate were calculated as 5.20 ± 0.28 and 21.00 ± 0.72 μg/mg, respectively. The enhanced antioxidant activity of the hydrolysate resulted from the opening and exposure of active amino acid residues, which were electron donors and reacted with free radicals to terminate the radical chain reaction. The TEAC value of the hydrolysate from freshwater clam was relatively lower than that from Pacific hake (*Merluccius productus*:

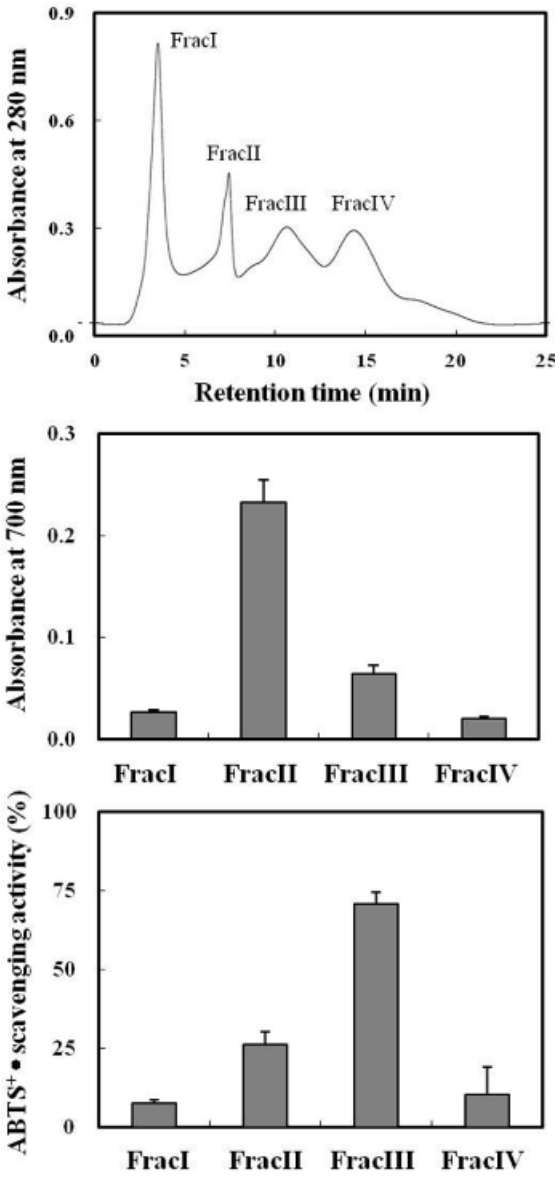

Figure 2. Gel-filtration chromatography of antioxidant peptides from the hydrolysate of freshwater clam extract. The separation was carried out on a Sephadex G-25 column using deionized water as the eluent. The antioxidant activities of the eluted peaks were determined by reducing power and $ABTS^{+\bullet}$ radical scavenging activity assays.

65.58 μg/mg) (Samaranayaka and Li-Chan, 2008). However, the antioxidant activity of the hydrolysate could be markedly improved via the purification of peptides.

Antioxidant peptides purified by gel-filtration chromatography

The hydrolysate was fractionated using Sephadex G-25. As shown in Figure 2, four major peaks were detected by the absorbance at 280 nm. The fractions associated with each peak (named as Frac I, II, III, and IV) were collected and lyophilized. Their antioxidant activities were evaluated by reducing power and $ABTS^{+\bullet}$ radical scavenging activity assays. Among these four fractions, Frac II had the strongest reducing power, whereas Frac III had the highest $ABTS^{+\bullet}$ radical scavenging activity. The absorbances of Fracs II and III at 700 nm were 0.233 and 0.064, respectively, at the peptide concentrations of 0.62 and 0.86 mM/ mM/(mg·mL), respectively. The TEAC values of Fracs II and III were measured as 58.84 ± 2.43 μg/mg and 158.04 ± 2.43 μg/mg, respectively. In addition, the IC_{50} value of Frac III against $ABTS^{+\bullet}$ was measured as 21.33 μg/mL and was sevenfold higher than that of the hydrolysate.

Using MALDI-TOF-MS, the molecular weight distributions of Fracs II and III were analyzed (Figure 3). Most peptides in Frac II were greater than 1000 Da, mainly ranging from 1000 to 2500 Da, whereas those in Frac III were below 1000 Da. These findings indicated that short peptides (between two and 8 amino acid residues) were the main components of Frac III. Considering the different mechanisms of the reducing power and $ABTS^{+\bullet}$ radical scavenging activity assays, peptides greater than 1000 Da evidently possessed better abilities to donate electrons, whereas those below 1000 Da more effectively eliminated free radicals. The higher radical scavenging activity of small peptides may be induced by steric effects given that smaller peptides have better access to the radicals. Similar observations have been obtained by other researchers. Wu et al. (2003) have found that mackerel hydrolysate with a molecular weight of 1400 Da possesses a stronger antioxidant activity than fractions whose molecular weights are 900 and 200 Da. Among enzymatic hydrolysates from purple sea urchin *(Strongylocentrotus nudus)* gonad, fractions below 1 kDa exhibit the highest DPPH radical scavenging capacity, whereas those ranging from 1 to 3 kDa show the highest reducing capacity (Qin et al., 2011).

Antioxidant peptides further purified by RP-HPLC

For further purification, Frac III with the highest radical scavenging ability was subjected to RP-HPLC and divided into nine major portions (P1 to P9) by the gradient elution of acetonitrile (0 to 36%) containing 0.1% TFA. Accordingly, the $ABTS^{+\bullet}$ radical scavenging ability of the nine major portions was evaluated. As shown in Figure 4, P5 exhibited a noticeable free radical scavenging effect, whereas the others had weak efficacies.

The molecular weight of P5 analyzed by MALDI-TOF-MS was similar to that of Frac III, especially in the low-mass region below *m*/*z* 500 (Figure 3, inset). This similarity can be attributed to the used matrix of CCA, considering that ions produced from the matrix of CCA generally appear in this low-mass region and interfere with the acquisition of useful data (Langley et al., 2007). The peak at *m*/*z* 568 may also correspond to a multimer

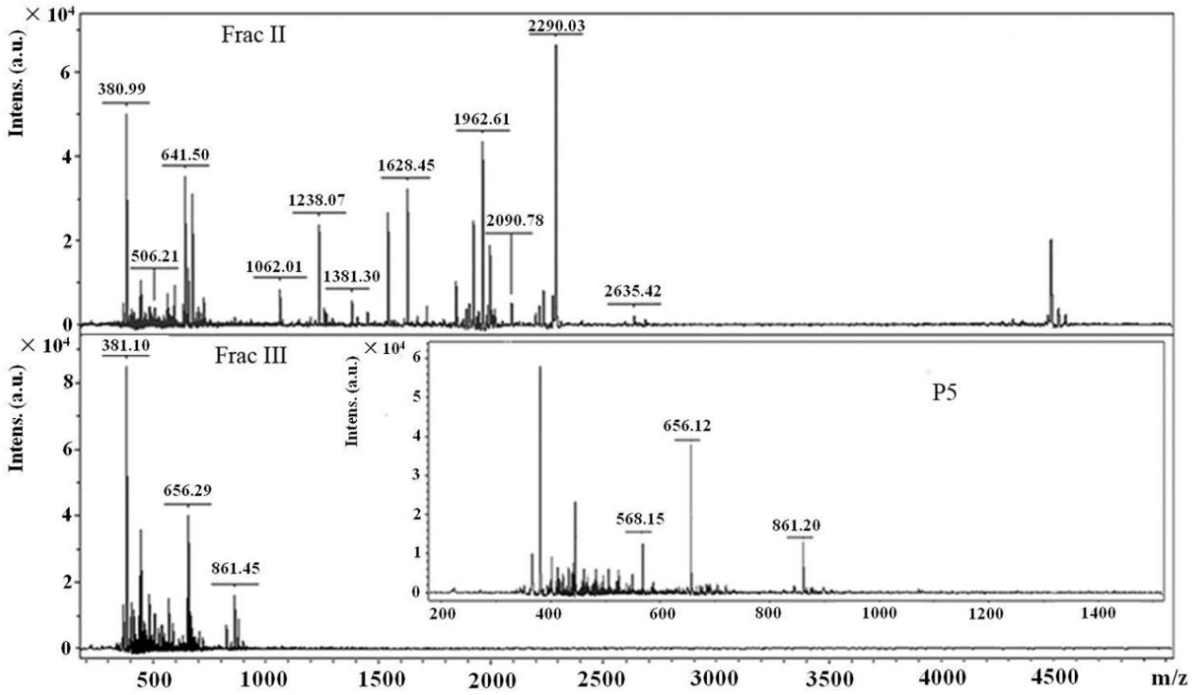

Figure 3. MALDI-TOF-MS spectra of Fracs II and III isolated from the hydrolysate of freshwater clam extract. Inset: MALDI-TOF-MS spectrum of P5 purified from Frac III.

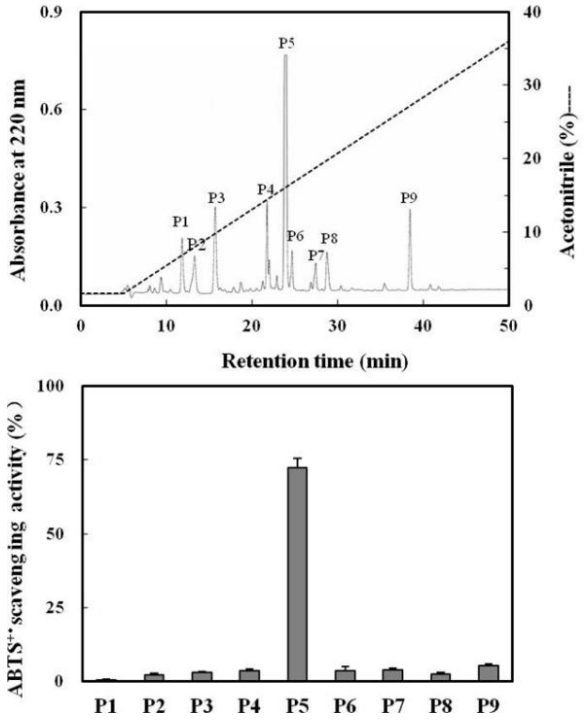

Figure 4. RP-HPLC chromatograms (TSK-gel ODS-80Ts column) of antioxidant peptides from Frac III. The elution was performed with the linear gradient of acetonitrile (0% to 36%) containing 0.1% TFA. The antioxidant activities of the eluted peaks were determined by the $ABTS^{+\cdot}$ radical scavenging assay.

matrix ion $(3HCCA+H)^+$ (Sönksen and Roepstorff 2001). Consequently, two major peptides in P5 were observed at the $[M+H]^+$ *m/z* values of 656 and 861 Da. The molecular weights of these peptides excellently agreed with previous studies. One such report is that on Ile-Glu-Phe-Phe-Thr-NH_2 isolated from the Australian red tree frog *Litoria rubella* (Steinborner et al., 1996), and another is that on a bradykinin-related peptide, Thr-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg, obtained from *Phyllomedusa hypochondrialis* (Brand et al., 2006).

The antioxidant activity of a peptide depends not only on its molecular size, but also on its chemical properties, such as hydrophobicity and the electron transferring ability of its amino acid residues. Although the antioxidant activities of the two reported peptides from frogs have not been studied, their amino acids Pro, Leu, and Arg have been confirmed to possess obvious antioxidant activities in other peptides (Chen et al., 1996). Phe has also been considered as an auxiliary antioxidant because it can render active oxygen stable via direct electron transfer, and donate protons to electron-deficient radicals (Jumeri and Kim, 2011). Based on this, the fraction of P5 purified from the enzymatic hydrolysate of freshwater clam extracts was expected to exhibit a strong antioxidant ability.

Conclusion

An enzymatic hydrolysate from freshwater clam extract was prepared and purified. Compared with the crude hot water extract, the hydrolysate exhibited a remarkably improved antioxidant activity based on reducing power and $ABTS^{+\bullet}$ radical scavenging capacity assays. The antioxidant activity of the hydrolysate was further improved by more than sevenfold via gel filtration purification and RP-HPLC.

The MALDI-TOF-MS results indicated that higher-molecular weight (1000 to 2500 Da) peptides in the hydrolysate had better reducing powers, whereas peptides below 1000 Da more efficiently scavenged free radicals. Therefore, antioxidant peptides could be prepared from freshwater clam extracts via enzymatic hydrolysis, and may be used as ingredients of food and nutraceuticals.

ACKNOWLEDGMENTS

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Biological control of apple gray mold by mixtures of *Bacillus Subtilis* and yeast isolates

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In this study the effect of biocontrol agents *Candida membraniciens* (isolates A4, A5), *Pichia guilliermondii* (isolate A6) and *Bacillus subtilis* (isolates B2, B6) were evaluated individually and in combination on gray mold of apple. Our results show that the antagonists were compatible when they were tested *in vitro*. Also, results show the spore germination of *Botrytis cinerea* decreased significantly by the combination of B2+A5 more than the other treatments. In the dual culture test, the combinations of B2+A5 and B6+A6 prevented mycelial growth of pathogen and that the combined application of the two agents was more effective in control of gray mold of apple *in vivo* than the application of each one alone. In different proportions antagonist test, when the inoculum size favored A5 was 70 in the mixed, the biocontrol could be improved. This study suggested that antagonists are more effective in biological control of apple gray mold when used in proper combinations than when each one is used alone.

Key words: Gray mold, biocontrol, mixtures antagonist, *Botrytis cinnerea*.

INTRODUCTION

Fruits and vegetables suffer significant losses from fungal diseases after harvest (Filonow, 1998). Postharvest losses of fruits and vegetables are high, ranging from 10 to 40% depending on the species. Among them gray mold of apple is created by wound-invading necrotrophic *Botrytis. cinerea Pers.:Fr* (Romano et al., 1983). Currently, Thiabendazole, is the main fungicide globally used for the control of postharvest fungal fruit decays of apple (Pusey, 1989). The fungicide is applied as the drench treatment before or after the cold storage. The development of resistance in fungal pathogens to fungi-cides and the growing public concern over the health and environmental hazards associated with high levels of fungicides have resulted in a significant interest in the development of alternative nonchemical methods for control of post harvest diseases (Gullino et al., 1994). Biological control using some antagonistic microorganisms is the safe method for control of postharvest pathogens (Yu and Zheng, 2006). Puesey, (1984) and Lima et al. (1997) claimed that considerable success was achived by utilizing antagonistic microorganisms for controlling postharvest diseases. In recent years many attempts are made to develop the efficacy of existing biological agents. There are many aproaches to achieve this goal through: Enhancing efficacy by addition of fungicides (Chand-Goyal and Spotts, 1996); salt (Gholamnejad and Etebarian, 2009); nutrients (El-Ghaouth et al., 2000a); chitosan (El-Ghaouth et al., 2000b);integration with physical means (Stevens et al., 1997); and finally using mixture of

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Abbreviations: OD, Optical densities; **NYDB,** nutrient yeast dextrose broth; **NYDA**, nutrient yeast dextrose Agar; **PDB,** potato-dextrose broth; **Ee,** expected effect; **LR**, lesion reduction.

antagonists **(**Janisiewicz, 1988). The mixture of biological control agents have many benefits: it can increase the constancy of biological control of apples in the field and later in storage (Leibinger et al., 1997), and also it can be effective to control many post hasvest diseases at the same time (Janisiewicz, 1996). Also suggested, the employment of more than one biocontrol agent caused to survive biological control in unsteady position (Guetsky et al., 2001). And, the combination of antagonists with different mechanisms of disease suppression might simultaneously suppress many pathogens (Guetsky et al., 2002). Several studies have tested different biocontrol strains in combination, for example Leibinger et al. (1997) showed that mixture of two yeast isolates and one bacterium controlled the postharvest pathogens *Pencillium expansum*, *B. cinerea* and *Pezicula malicorticis* on apple. Calvo et al. (2003) showed that mixing different isolates of yeast *Rhabdotorula* and *Cryptococus* were considerably more effective in controlling *P. expansum* and *Botrytis cinerea* on Red Delicious apple fruits. Guetsky et al. (2001) combined *Pichia guilliermondii* with *Bacillus mycoides* to improve control of gray mold of strawberries by *B. cinerea* in storage. In this study, we applied different mixture of antagonists for controlling postharvest gray mold of apples.

MATERIALS AND METHODS

Atagonists

The antagonistic yeasts *Candida membranifuciens* (A4 & A5) and *Pichia guilliermondii* (A6) were obtained from plant pathology laboratory of Abureihan Campus, University of Tehran. The isolates were identified previously in Centralbureau voor Schimmelcultures (CBS). For biocontrol experiments, yeasts were grown on PDA media for 24 to 48 h at 25°C before use, then cultures were harvested by a bacteriological loop and resuspended in sterile distilled water and cell concentrations were adjusted with sterile distilled water to 1×10^7 cell/ml.

The *Bacillus subtilus* isolates B2 and B6 were also obtained from plant pathology laboratory of Abureihan campus, university of Tehran. *B. subtilus* isolates were grown in 250 ml erlenmeyer flasks containing 50 ml of NYDB (*Nutrient Yeast Dextrose Broth)* on a rotary shaker (150 rpm) at 25°C. Then the cultures were harvested by centrifugation and resuspended in sterile distilled water and the population of the bacterial cells was adjusted to 10^8 cells/ml (optical densities (OD) 0/05 transmittance at 590 nm). For biocontrol experiments suspensions of the yeast and bacterium at each concentration were mixed in proportions of 50: 50 (v/v).

Pathogen

B. cinerea was isolated from decayed apple. The fresh cultures were grown on PDA plate at 25°C before use then spore suspension were prepared from the spourulating edges of 10 day old culture with a bacteriological loop, then suspending them in sterile distilled water containing 0.05% (v/v) Tween 80. Spore concentration was determined with hemacytometer and adjusted with sterile-distilled water to 1×10^5 spore/ml.

Plant

Golden Delicious apples were obtained from organically grown position in orchard in Damavand, Tehran.

Compatibility of biocontrol agents with each other

The compatibility of the biocontrol agents (yeast and bacteria isolates) with each other was tested following the methods of antibiosis and mixed culture tests, *in vitro*.

Antibiosis test

15 mm diameter disks from 5 days old NYDA cultures of the three yeast isolates (A4 , A5 , A6) were cut and then they were spotted onto center of one surface dry plate PDA (9 cm plate). The plates were incubated for 72 h at 25°C. Following incubation, the plates of the yeast isolates were oversprayed with a suspension (10^8 cell/ml) of *Bacillus subtilis* isolates (B2, B6). The plates were incubated for 48 h at 25°C. Then, the plates were examined for the presence of inhibition zones. All treatments consisted of four replicates (Wilson and Lindow, 1994).

Mixed culture test

To study interaction of the antagonists *in vitro*, the cultures of yeast and bacteria were resuspended in water (adjusted to 10^8, 10^7 cell/ml bacteria and yeast isolates respectively) and 100 micro liter of these suspensions were added individually to 10 ml Erlenmeyer flasks containing 5 ml of NYDB. Another set of flasks was inoculated with a combination of both organisms in the proportion of 50:50. The flasks were incubated on the shaker at 150 rpm and 24°C for 30 h. Samples from the flasks were taken at 15 and 30 h intervals and were plated on PDA or PDA supplemented with 25 mg of streptomycin per liter for recovering bacterial and yeast cells respectively. This samples were inoculated with mixture of the antagonists were plated in duplicate, once on regular PDA to recovery of the bacterial antagonist and the second on PDA supplemented with streptomycin sulfate at 25 mg/lL, which inhibited the bacterium but allowed the yeast to grow. Finally, for evaluate the yeasts population, the colonies were counted with a colony counter after incubation for 48 h. All treatments consisted of three replicates (Janisiewicz and Bors, 1995).

In vitro antagonism

The biocontrol agents were tested alone and in different combinations for their effectiveness against the mycelial growth of *B. cinerea* by the dual culture technique (Etebarian et al., 2005). Half of the agar surface were streaked with 100 µl suspension of 10^7 Cell/ml of the yeast isolates (A4, A5 and A6), 10^8 cell/ml of *B. subtilis* isolates (B2 and B6) and an equal their mixtures (50:50). After 2 days of incubation, a mycelial disc (9 mm) of *B. cinerea* of ten-day old culture was placed on the other side of each plates. For control, the medium was incubated with the pathogen alone. Four replications were carried out for each treatment; the dishes were incubated at 25°C for 7 days and the percent growth inhibition was calculated using the formula: $n = (a-b)/a \times 100$, where *n* is the percent growth inhibition, *a* is the colony area of *B. cinerea* for the control and *b* is the colony area of *B. cinerea* treated with different combinations of the yeasts or the bacteria.

Effects of alone or the combination treatments of antagonists on spore germination of pathogen were carried out in PDB culture. The 100-µl of 1×10^8 Cell/ml of the washed cell suspension of bacteria

isolates (B2, B6) and 1 × 10^7 Cell/ml of the yeast isolates (A4, A5, A6) and the sterile distilled water as the control were added into 10 ml glass tube containing 5 ml PDB respectively. Another set of glass was inoculated with same quantity of a combination of both antagonists in the proportion 50:50. At the same time, aliquots 100 µl of spore suspensions 1 × 10^5 spores/ml of *B. cinerea* were added into each tube. After 20 h incubation at 25°C, at least 100 spores per replicate were observed microscopically to determine germination rate on a rotary shaker (150 rpm). All treatments consisted of four replicates (Droby et al., 1997).

In vivo biological control studies

Fruits were surface-disinfected with sodium hypochlorite (0.1%) for 2 min and washed two times by immersion in distilled water,Then allowed to air-dry and wounded 3 × 3 × 3 mm in three places on the equator of the fruit with a sterilized needle , then 40 µl of suspension 10^8 and 10^7 Cell/ml of bacteria and yeast isolates respectively (singly or their mixtures 50:50) was pipeted in to apple wounds. after 24 h , the treated wounds were inoculated with 20 µl of *B. cinerea* spore (1×10^5 spore / ml). The lesion diameters were determined 15 and 30 days after storage at 20 and 4°C in 95% relative humidity. Each apple constituted a single replicate and each treatment was replicated four times. Wounded apples were distributed into five sets: (1) Non-treated apples (control), (2) Apples treated with isolates yeast or bacteria alone (antagonist), (3) Apples inoculated with *B. cinerea* alone (pathogen), (4) Apples treated with isolates yeast or bacteria individual and then inoculated with *B.cinerea* (antagonist+ pathogen) (5) Apples treated with mixture of antagonists and inoculated with pathogen (mixture of antagonists + pathogen). The percentage of lesion reduction was estimated according to the formula:

$$LR = (Dc - Dt) / Dc \times 100$$

Where, Dc and Dt are, the lesion diameters of the control and treated apples respectively (Etebarian et al., 2005). Limpel's formula also was used to determine the presence of synergistic or antagonistic interactions between the antagonists in mixtures:

$$Ee = X + Y - X.Y/100$$

Where, *Ee* is the expected effect from additive responses of two inhibitory agents and *X* and *Y* are the pecentages of lesion reduction for each agent used alone. If the combination of the two agents produces any value of inhibition greater than Ee, then synergism exists, on the same basis, if the observed effect (pecentage of lesion reduction) would be less than the expected effect, then the mixture exhibits antagonism (Limpel et al., 1962).

Biocontrol test *in vivo* in the various proportions of mixture antagonists

In vivo biological control studies when two treatments as B2+A5 and B6+A6 were combined at approximately equal biomass (50:50), they were consistently compaierd to the other combinations. The apple fruits were disinfected and wounded as described above, then aliquots 40 µl suspensions at 10^7 and 10^8 cell/ml yeast and bacterium were mixed in proportions from 0:100, 30:70, 70:30, 50:50, 60:40, 40:60, 100:0. This proprtions were pipetted into wounds after 24 h, 20 ml of 10^5 spores/ml suspension of *B. cinerea* were inoculated into each wound. Treated fruits were stored at 20°C for 15 days or 4°C for 30 days. The lesion diameter was recorded afterwards. There were three replicate trials of 5 fruits per treatment with complete randomization (Janisiewicz and Bors, 1995).

Recovery of the antagonists

Two combinations B2+A5 and B2+A6 was conducted to recover the antagonists. Three wounds per fruit were made as described above, and then 40 µl of each antagonist alone and an equal mixture (50:50) was pipetted to each wound. After 24 h, the tested wounds were inculated with 20 µl of *B. cinerea* spore (1 × 10^5 spore/ml).The fruit apples were stored for 30 day at 4؛C, then the fruit samples were taken to determine antagonist populations after 15 and 30 days of storage. The wounded area was removed with a cork borer (no. 5) and placed in a sterile mortar, and ground with pestle. For each apple, recovered cell suspensions from the three wounds were diluted 10^3. 10^4 and 10^5 Serial tenfold dilution and were plated on PDA or PDA supplemented with 25 mg of streptomycin per liter for recovering bacterial and yeast cells, respectively. Samples from flasks inoculated with the mixture of the antagonists were plated in duplicate on PDA and PDA plus streptomycin. The colonies were counted with a colony counter after incubation for 24 and 48 h for the bacteria and yeast, respectively (Teixido et al., 2000).

Data analysis

The *in vitro* and *in vivo* assays were analyzed by an analysis of variance (ANOVA) with SAS Software (SAS Institute, version 9.0, Cary, NC). All assays were carried out with three/four_replicates. Statistical significance was judged at the level $p < 0.05$. When the analysis was statistically significant, Duncan's Multiple-Range Test (SSR Test) was used to test mean separations among mean values of each treatment (Little and Hills, 1978).

RESULTS

Compatibility of biocontrol agents with each other

In the test antibiosis, the isolates which grew togother were compatible, but isolates that were separated by an inhibition zone were incompatible. From these results none of the antagonists showed antibiosis effect on each other. Both *B. subtilis* isolates (B2 and B6) and the yeast isolates grew together and it indicated that these isolates were compatible.

Interaction of the antagonists *in vitro* showed the mixtures of B2+A4, B2+A5, and B2+A6 the growth of yeast isolates wasn't effected by the presence of *B.subtilis* isolate B2 (P≤0.05). In NYDB inoculated with yeast isolate alone, the yeast population increased in all flasks after 15 h, and the cells increased until end of the experiment. However, the increases of the mixtures yeast isolate (A4, A5 and A6) with the bacteria, almost were equal with alone antagonists after 30 h *(*Figure 1 A). Also, in the mixtures of B6+A4, B6+A5 and B6+A6, the growth of yeast isolates was not affected by the presence of *B. subtilis* isolate B6 (P≤ 0.05). Results showed that the populations of the yeast isolates inoculated in mixture of *B.subtilis* increased in all flasks after 30 h. In the other hand, the application of the yeast population alone increased in all flasks after 15 h, and then it increased until end of the experiment (Figure 1 B).

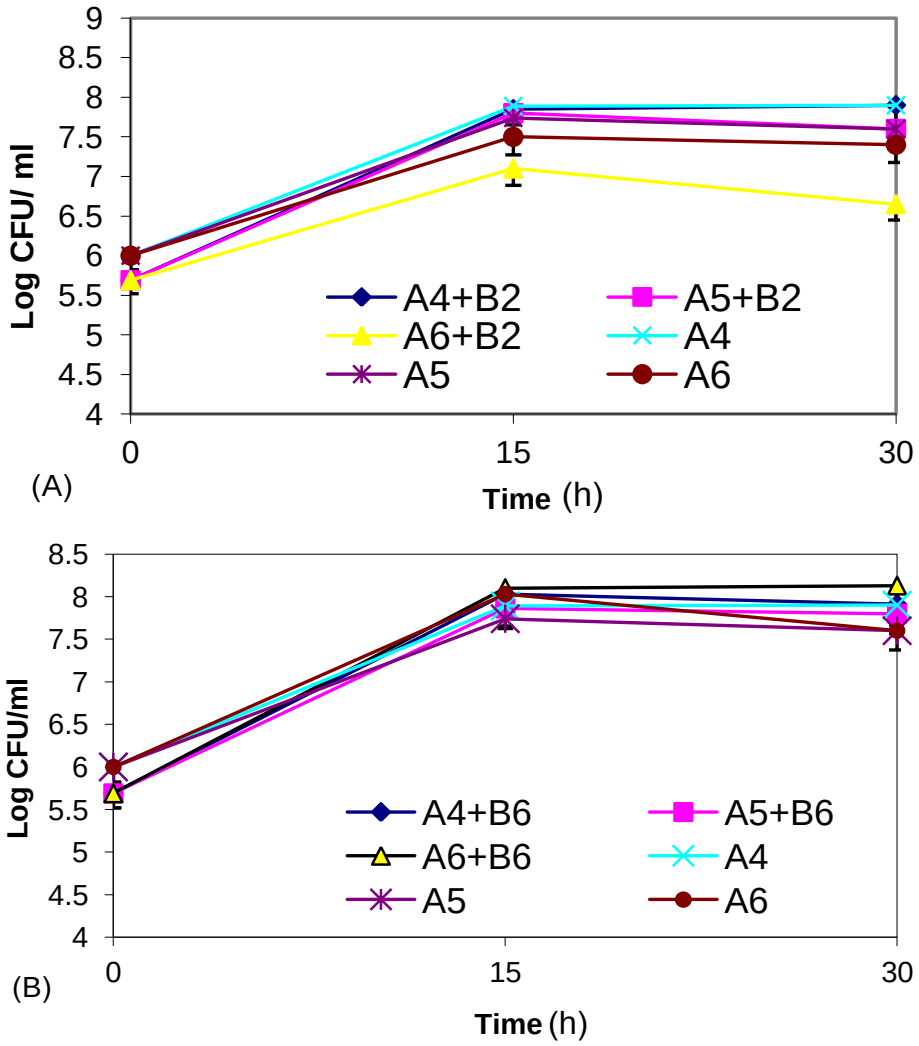

Figure 1. Population dynamics of yeast isolate (A4, A5, A6) by the presence of *B.subtilis* B2 (A), B6 (B) alone or in mixtures in NYDB. The samples were collected at 15 h intervals. Bars represent standard error.

In vitro biological control studies

Results show that all treatments reduced significantly the mycelial growth of *B. cinerea* (P≤0.05). The tretment B2+A5 had the highest percent of inhibition at 53.26%, if the single isolates inhibition A4, A5, A6, B2, B6 were 20.5, 21.28, 32.21, 40.54 and 39.73 %,respectively. The percent inhibition of *B. cinerea* of other treatments such as B2+A4, B2+A6, B6+A5 and B6+A4 was equal to, or further than the inhibition achieved by the isolates applied alone (Table 1).

Spore germination of *B. cinerea* in PDB culture was inhibited strongly in the presence of active cells of antagonists and their mixtures (P≤0.05). At the end of 20 h incubation at 25°C, the spore germination of *B. cinerea* incubated with the combination B2+A5 was 10% while the spore germination of the other combinations were equal to, or further than the isolates applied alone (Table 2).

In vivo biological control studies

The results show that wound diameter of all treated fruits were significantly lower than the untreated controls (P≤0.05). The lesion diameters of the biocontrol combinations B2+A5, B6+A6, B6+A4 on treated fruits were 5/23, 7/43, 11/66 mm, respectively, after storage at 20°C for 15 day, that provided better control than the antagonist applied alone B2, B6, A5 and A4 that had a lesion diameter of 15/06, 15/22 13/57 and 12/99 mm respectively.

On the other hand the lesion diameter, in the apple fruits treated with the combinations of yeast and bacteria, the lesion diameter of fruits with the best combination such as B2+A5, B6+A6 and B6+A5 was 3/48,3/87 and

Table 1. Effectiveness of mixture *Bacillus subtilis* and yeast isolates in inhibiting mycelial growth of *Botrytis cinerea.*

Treatment isolateds[1]	Mycelial growth of the pathogen (mm)	Inhibition over control (%)
A4	31.96$^{b\ 2}$	20.5
A5	31.65^{bc}	21.28
A6	27.25^{dc}	32.21
B2	23.9^{de}	40.54
B6	24.23^{de}	39.73
B2+A4	22.94^{def}	42.64
B2+A5	18.79^{f}	53.26
B2+A6	31.48^{bc}	21.7
B6+A4	22.65^{dfe}	43.66
B6+A5	23.53^{de}	41.47
B6+A6	22.07^{fe}	45.09
Control	40.21^{a}	0.0

[1]*Bacilus subtilis* isolate B2 and B6 , *C. Membranifuciens* (A4 and A5), *P. guilliermondi* (A6). [2]Values are the means of four replications. Means followed by a common letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Table 2. Effectiveness of mixture *Bacillus subtilis* and yeast isolates in inhibiting Spore germination of *Botrytis cinerea.*

Treatment isolateds[3]	Spore germination[1] (%)
A4	59$^{ab\ 2}$
A5	45.33^{cb}
A6	13de
B2	14.66^{de}
B6	19.66^{de}
B2+A4	28^{dc}
B2+A5	10^{e}
B2+A6	55.33^{ab}
B6+A4	30^{cd}
B6+A5	21.66^{de}
B6+A6	19.33^{de}
Control	82.33^{a}

[1]Germination rate were measured microscopically after 20 h incubation at 25°C in PDB. [2]Each value is the mean of four experiments. Values in each column followed by different letters are statistically different according to Duncan's multiple range test at(P≤0.05). [3]*Bacilus subtilis* isolate B2 and B6, *C. Membranifuciens* (A4 and A5), *P. guilliermondi* (A6) .

5/16 mm, respectively, wheras the biocontrol isolates applied alone such as B2, B6, A6, A5 and A4, it was only 13.29, 10/83, 8/97,11/67 and 16/01 respectively, after storage for 30 day at 4°C (Figure 2 A and B).

The expected effect of Ee, accordance to the formula Limpele, showed that B2+A5 and B6+A6 compounds at 4°C and B2+A5 at 20°C had a synergistic effect in controlling gray mold. Its reason is that the calculated LR was more than Ee.In other compounds, the number of calculated Ee was more than the number of suppresions percent LR, it showed that these compounds had the antagonistic effects against gray mold (Table 3).

Biocontrol test *in vivo* in the various proportions of mixture antagonists

The results showed that the different proportion of the antagonists affected the lesion diameter, significantly (P≤0.05). Also, the results show, the mixture of B2+A5 (proportion of 30% of bacterium and the proportion of 70% of yeast), reduced the lesion diameter to 5 mm, this compounds didn't have significant difference with other mixtures (with different proportions antagonists) expect of B2(70)+A5(30) at 20°C. Also, the results showed that there is a significant difference between treatments at 4°C (P≤0.05). The treatment of B2+A5 with 50:50 ratio for yeast and bacteria, and other mode with 30:70 ratio for bacreria and yeast was more effective than other proportions and the antagonists applied individually on lesion development, respectively (Figure 3 A and B).

Recovery antagonists

The results showed that both *B. subtilis* (B2) and yeast isolates (B6) multiplied rapidly in apple wounds in the presence of *B*. *cinerea*. At the start of the experiment (time 0), the number of per yeast and bacterium strain was 2×10^5 CFU/ml for mixture of B2+A5, also the population of *C. Membranifuciens* (A5) and *B. subtilis* (B2) increased from 1.1×10^7 and 6.31×10^6 CFU/wounds, respectively at 15 d. But after 30 days incubation at 4°C, the yeast and bacteria population began to decline. The comparison between logarithm of mixed antagonists and their individual applications showed that the population of B2 and yeast A5 in mixture is more than they applied alone. Indeed the results showed that a positive interaction probably occurred between these antagonists in mixture and in this position, the quantity of both antagonist's cell is more than when they applied alone. The study of antagonist's population in mixture of B2+A6 have been showed that the population logarithm of alone antagonists as more than their mixture in all test period, this can be due to an antagonism effect among them (Figure 4 B).

DISCUSSION

In earlier studies the multiple strain mixtures of bio-

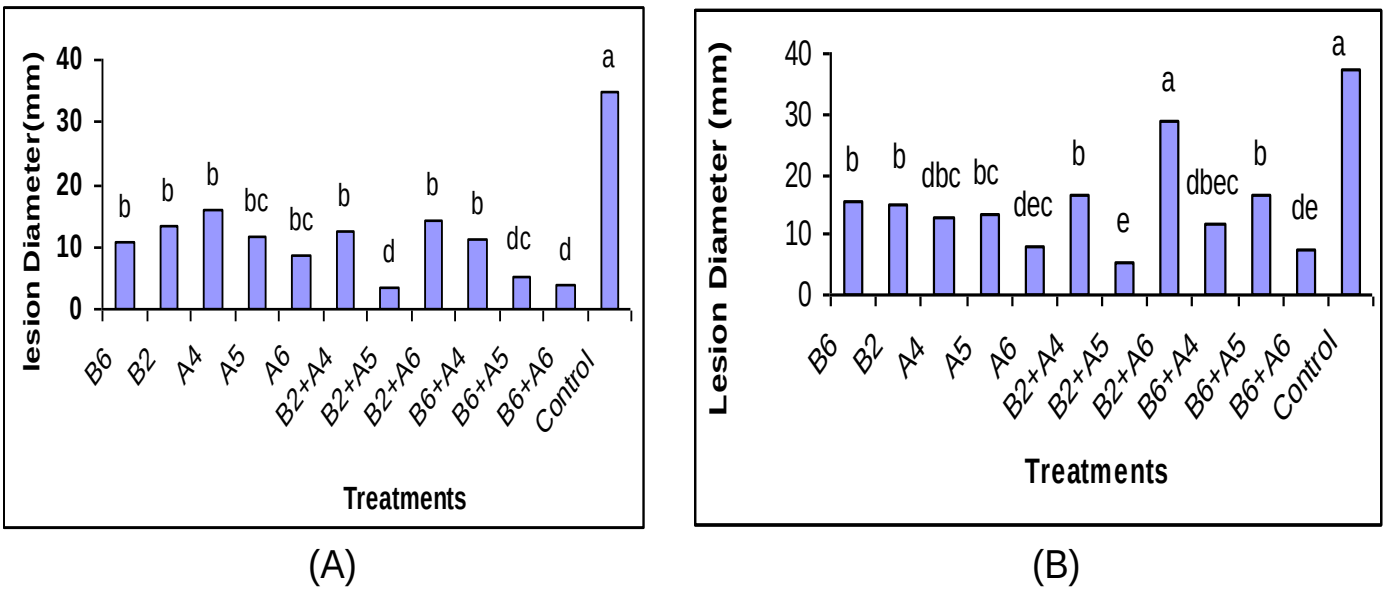

Figure 2. Inhibition of gray mold in apple fruits .the lesion diameters were measured after 30 days incubation at 4°C (A), and 15 days incubation at 20°C (B). Different letters in the assay indicate significant differences between means according to Duncan's multiple range test ($p \leq 0.05$).

Table 3. percentage of lesion reduction of mixture *B.subtilis* isolate (B2,B6) and yeast isolates of (A4,A5 and A6) and expected effect (Ee) of mixtures according to Limpel's formula.

	20°C		4°C	
Treatment isolateds[2]	**lesion reduction (LR) %**	**Ee**	**lesion reduction (LR) %**	**Ee**
A4	65.14^{bc1}		54.24^{c}	
A5	60.56^{bc}		66.64^{bc}	
A6	77.93^{a}		72.22^{abc}	
B2	59.63^{bc}		62^{c}	
B6	55.55^{dc}		69.09^{cb}	
B2+A4	66.59^{b}	91.09	64.38^{c}	90.58
B2+A5	86.94^{a}	85.07	90.02^{a}	87.32
B2+A6	28.41^{e}	85.92	58.96^{c}	82.61
B6+A4	59.32^{dbc}	90.18	68.32^{cb}	92.32
B6+A5	50.21^{d}	82.46	85.23^{ab}	89.66
B6+A6	80.01^{a}	84.50	88.93^{a}	85.83

[1]Each value is the mean of three experiments. Values in each column followed by different letters are statistically different according to Duncan's multiple range test at (P≤%05). [2]*Bacilus subtilis* isolate B2 and B6, *C. Membranifuciens* (A4 and A5), *P. guilliermondi* (A6) .

control agents have been employed successfully against plant pathogens (Janisiewicz, 1996). Several authors have suggested that combinations of bio-control agents have to be compatible with each other for more consistent results of biological control (Raaijmakers et al., 1995). According to *in vitro* antibiosis test the selected antagonist strains do not have any antibiotic element against each other. Interaction of the antagonists *in vitro*, also showed that the growth of yeast isolates were not affected by the presence of *B.subtilis* B2 and B6 isolates in NYDB culture. It indicated that the *B.subtilis* isolates B2 and B6 were compatible with yeast strains A4, A5 and A6. Tillugavati et al. (2007) so as to affirm the compatibility between two bacterium (*Bacillus* and *Psudomonase*) and one fungus (*Trichdema*) in mixture, used the dual culture test of these antagonists with pathogen. They concluded that these three biocontrol agents create more inhibition of growth of mycelia of *B. cinerea* than single antagonists used alone, it was suggested its reason is the compatibility between bio-

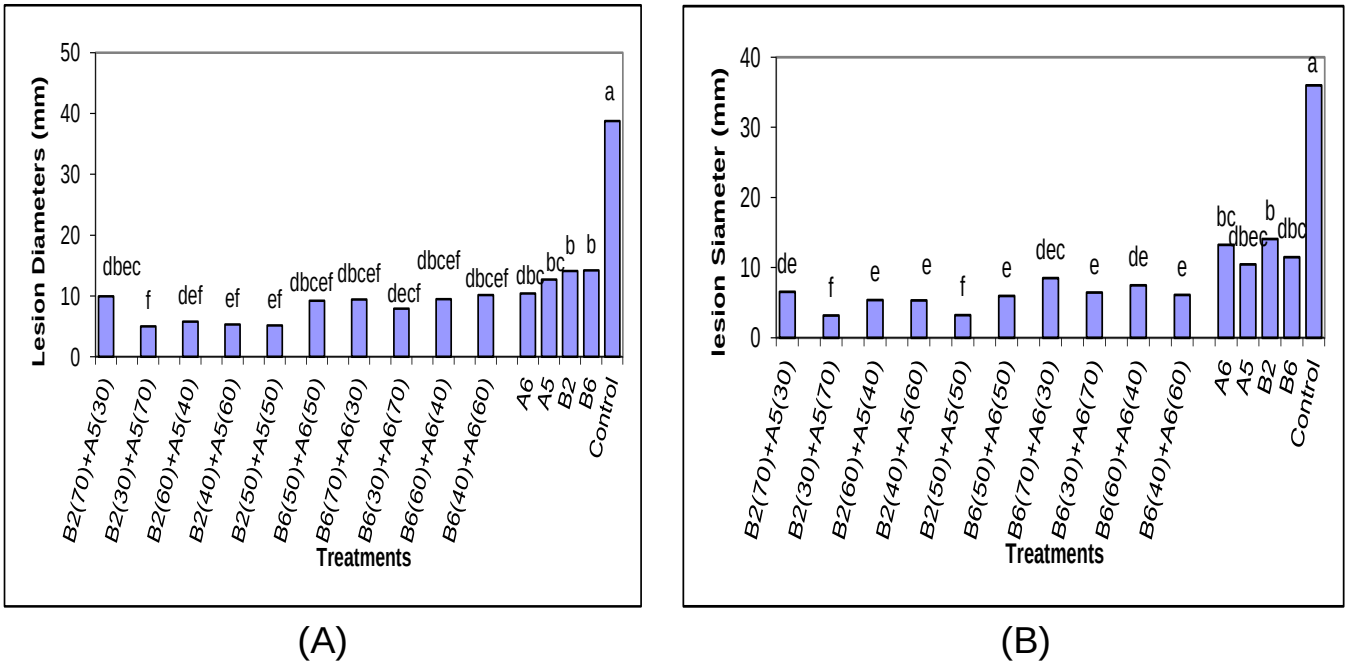

Figure 3. Inhibition of grey mold in apple fruits as affected by various proportions(0:100, 30:70,70:30,50:50,60:40,40:60,100:0) treatments of the antagonist. lesion diameters were measured after 15 days incubation at 20°C (A), and 30 days incubation at 4°C (B). Values followed by different letters are significantly different according to Duncan's multiple range test at $p \leq 0.05$.

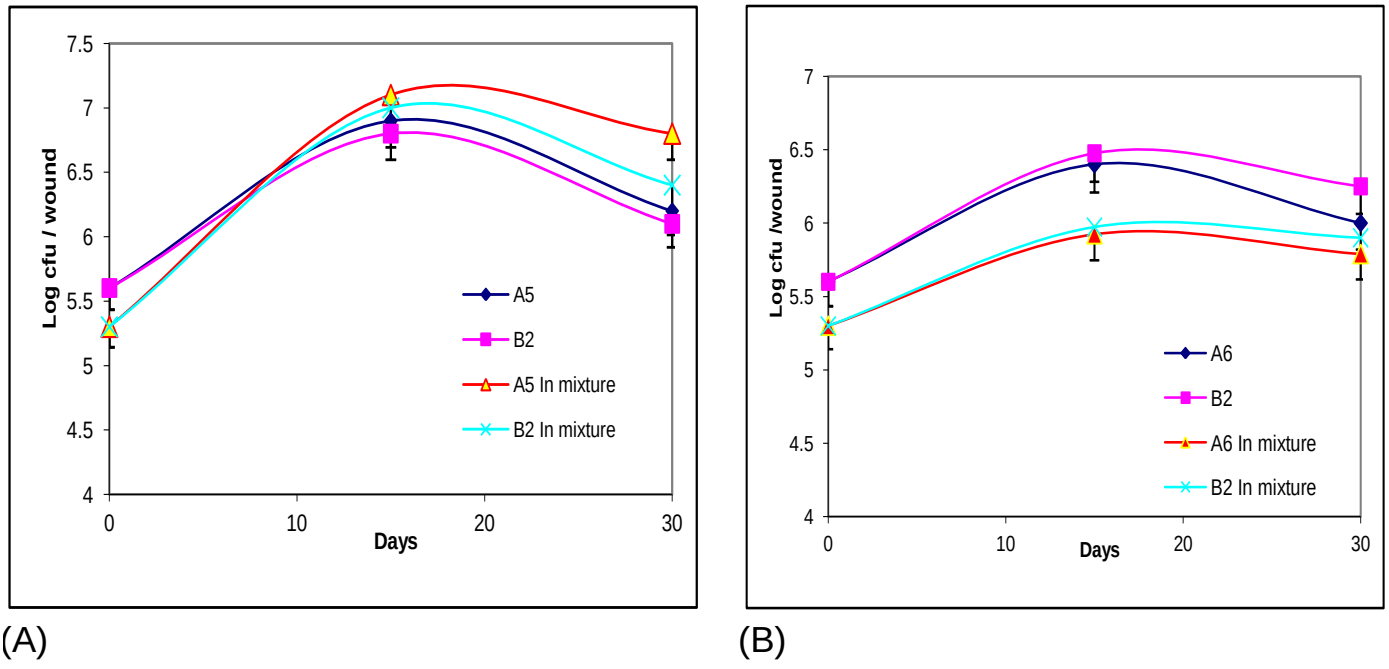

Figure 4. Population dynamics in wounds of Golden Delicious apples inoculated with *B.subtilis* B2 and *C.Membranifuciens* A5 isolate (A) and *B.subtilis* B2 and *Pichia guilliermondi* A6 isolate (B) the individually or in a 50:50 mixture and incubated at 4 °C. Bars represent standard error.

control agents. In our experiments, the compounds of B2+A5 and B6+A6 created the most inhibition of the growth of mycelia and spore germination of *B. cinerea*; this can be due to the compatibility between biocontrol

agents with each other. The combinations of B2+A5 and B6+A6 and a less extent B2+A4, B6+A4 and B6+A5, controlled apple gray mold more effectively when they were used alone. In addition, Limpel's formula (Limpel et al., 1962) and population size of yeasts allowed us to evaluate the interactions between yeast and bacteria isolates when they were used in mixtures. According to Limpel's formula the *B. subtilis* (B2) and *C. membranifaciens* (A5) mixture showed synergism against *B. cinerea*, because the LR% was calculated higher than Ee. Dynamic of populations, also explained that both *C. membranifaciens* A5 and *B. subtilis* B2 reached a higher cell concentration when they used in a mixture mode instead of using alone. These results agree with the findings of Janisiewicz and Bors who said carrying capacity of the wounds could be greater than the population of the single antagonists and they mentioned that another antagonist may caused to increase the population of the antagonists in the wounds to increase more improves of biocontrol (Janisiewicz and Bors, 1995). Also, they found that screening of specific amino acids may specifically enhance the growth of the antagonists but had no effect on pathogen. It was when the specific nutrient is available, the growth of the antagonist which metabolize it enhance, so both antagonists cannot compete for this nutrient and it creates desirable position for growth of both antagonists**.** Also, this caused even greater depletion of essential nutrients for development of *B.cinerea* and eventually better biocontrol. These results also agree with the findings of Calvo et al. (2003) and Janisiewicz (1996). In a more comprehensive study Gutsky et al. (2002) showed types of biocontrol mechanisms *B. cinerea* on strawberry by mixture of yeasts (*Pichia guilermondii*) and a bacterium (*Bacillus mycodes*) by scan of Electron Microscopy, they concluded that in the presence of yeast, some of *B. cinerea* spore cannot germinate, They indicated that *P. guilermondii* competed with *Botrytis cinerea* for glucose, sucrose, adenine, histidine, and folic acid. It was found that the half of spores of *B. cinerea* hydrolyzed fully in presence of *B. mycodes*. They suggested that these observations may be due to the presence of cell wall hydrolyzing enzymes. But when the mixture of two biocontrol agents was used, the spores were hydrolyzed with more intensity. Likewise, they expressed when both biocontrol agents were applied in a mixture, their activity reflected the sum of the biocontrol mechanisms. Most of researches on the mixtures of biocontrol agents showed that combinations of antagonists caused to improve biocontrol. But, studies show that some combinations of biocontrol agents could not improve suppression of disease compared with the separate antagonists (Thilagavathi et al., 2007; Leibinger et al., 1997). In this study, the mixture of B2+A6, had an equal or/and lower control than the separate antagonists. This may be due to an incompatibility between *B. subtilis* isolate B2 and *P.guilliermondi* isolate A6. Also, Limpel's formula showed that the interaction between them is antagonism. Morever, the dynamic of populations of B2 has a negative effect on the growth of A6 and application of isolate A6 in alone mode is more effective than in the mixture mode. Results of compatibility of biocontrol agents with each other *in vitro*, demonstrated that at least inhibitory substances (antibiosis) are not involved, probably. However many same studies showed that the basis of this incompatibility is unknown (Schisler et al., 1997).We can compare these results with Leibinger's test that saied a mixture of two yeast isolates and one bacterium controled the post-harvest pathogens *Penicillium expansum*, *B. cinerea* and *Pezicula malicorticis in vivo*. In their test the population size of yeast isolate *A. pullulans* reduced when it was applied in combination with *B. subtilis*, they reported that the basis of this incompatibility could result from the production of antibiotic compounds by *Bacillus subtilis* (Leibinger et al., 1997). From the test of different proportions of antagonist in B2+A5 mixture, we can understand that biocontrol would be improved when we use more inocoulom yeast A5 with 70%, its reason could be more colonization of yeasts in apple's wounds. It shows that yeasts, for control of the pathogens, have stronger mechanism than bacterium that include competition for nutrients and space (Leibinger et al., 1997). Calvo et al. (2003) showed that the gray mold in mixture of *Rhodotorula glutinis* SL1 and SL30 with inocoulom 1:2 for SL1, is controlled more better than proportion 2:1 for SL30. They concluded that the control of disease is depended on the size of inocoulom of antagonists in mixture. In this reserch, we showed that application of antagonists in combination mode is more effective than the application used alone, for a period of 30 days at 4°C and a period of 15 days at 20°C. The adaptation of these strains to a wide range of temperature (4 to 20 C°) provides great potential for this antagonist mixture for control of post-harvest diseases on apples in storage and transportation conditions. We hope that other biologists, physicians and agronomists work together for culturing and formulating of these agents and make commercial using of these antagonist microorganisms in the near future.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Food expenditure and household welfare in Ghana

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The main objective of this study was to investigate the determinants of household food expenditure and its effects on welfare. As a result of potential simultaneity between food expenditure and welfare, a simultaneous equations model was estimated using the two-stage least squares method. The findings confirm the theoretical and empirical evidences that households reduce the percentage share of their food expenditure as they become richer. Also, increases in the food budget share lead to a reduction in welfare. Different households which spent greater percentages of their incomes on food were as follows: female headed households; households headed by the aged; households whose heads had little or no formal education; households whose heads were married; smaller households; rural households; households in the forest and savannah belts; and households living farther from the nation's capital. Also, welfare was greater for the following households: female headed households; households headed by the aged, households whose heads had formal education, smaller households, households who owned assets; households living in the urban centres, as well as those living closer to the nation's capital. Households that must be targeted for support include male-headed households, households headed by the relatively young, larger households, rural households and households farther from the nation's capital, including those in the savannah belt.

Key words: Food expenditure, Ghana, households, two stage least squares, welfare.

INTRODUCTION

The Food and Agriculture Organization (FAO, 2011) noted that even though world food supply is enough to feed the population, there are profound disparities across countries, towns and households, in terms of access to

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Abbreviations: 2SLS, Two stage least squares; **BECE,** Basic Education Certificate Examination; **FAO,** Food and Agriculture Organization; **GSS,** Ghana Statistical Service; **LCH,** life cycle hypothesis; **MDG,** millennium development goal; **MSLCE,** Middle School Level Certificate Examination; **OLS,** ordinary least squares method; **PIH,** permanent income hypothesis; **SSA,** Sub-Saharan African; **UNDP,** United Nations Development Programme; **UNEP,** United Nations Expenditure Programme; **VSCE,** Vocational School Certificate Examination; **WFP,** World Food Programme; **WHO,** World Health Organization.

food. For instance, in 2010, while the average caloric intake per person per day in least developed and developing countries were 2,120 and 2,640 kcal, respectively, that of developed countries was 3,430 kcal. The FAO (2012) stressed that despite the considerable efforts taken to curb global hunger, 925 million people were undernourished in 2010, while the number of obese people rose to 1.5 billion in 2008. The World Health Organization (WHO, 2011) noted that these discrepancies call for much concern.

The irony is that while the rich spend more on food than the poor in absolute terms, the latter allocate high proportions of their income to food consumption with smaller portions left for the consumption of high quality non-food items (Engel, 1857) necessary for a higher standard of living. The over consumption of the rich and the under nourishment of the poor have significant health, economic and environmental implications (UNEP, 2012).

The main objective of this study was to investigate the socioeconomic determinants of food consumption and the effects on the living standards of Ghanaian households. Specifically, we sought to investigate the extent to which the Engel's law applies to the consumption behaviours of Ghanaian households.

Economic performance of the Ghanaian economy

Over the past two decades, the Ghanaian economy is viewed as a model of development. The country has experienced strong and sustained economic growth in a relatively stable and democratic political environment. Ghana became the first African country to achieve the first Millennium Development Goal (MDG) by halving the poverty of the 1990s even before the targeted year of 2015 (UNDP, 2008). Between 2000 and 2010, Ghana's growth rate at 4.9% was higher than the sub-Saharan African (SSA) average, and more than twice the growth rate of a decade earlier. Similarly, Ghana was more effective in translating the economic growth into poverty reduction than her counterparts in the developing world. However, the Ghana Statistical Service (GSS, 2007) reported that even though poverty has reduced since the 1990s, inequality has not changed. Poverty is the main cause of food insecurity (WFP, 2012). In its comprehensive food security and vulnerability analysis, WFP (2012, p.17) found that "poorer households have lower levels of education, spend a larger share of their limited means on purchasing food, have smaller harvests, and are more often buying their staple foods when the market prices are highest when compared with wealthier households".

Household expenditure in Ghana in 2006/2007

In 2006/2007, the average annual household expenditure in Ghana was GH¢1,918.00 whilst the mean annual per capita consumption expenditure was GH¢644.00.

Food expenditure accounted for 40% of total household expenditure, while the imputed value of own-produced food consumed by households represents a further 10.5%. At the time of the survey, Ghanaian households were spending on an average, about GH¢2,680 million per annum on food (including non-alcoholic beverages). This represented about a third of the total expenditure while non-food expenditure represented about 70% of the total household expenditure.

Food accounted for about half of the total expenditure of households in the highest quintile and also formed about 60% of the expenditure of households in the lowest quintile. In the localities, households in urban centres spent about 44% on food (actual and imputed), while households in the rural areas spent more than 60% on food. In terms of regions, household expenditure represented more than 70% of the budget in Volta, followed by the Northern Region (65.2%). The figure for the Greater Accra was 40%.

MATERIALS AND METHODS

Theoretical framework: Consumption theory

The theory of consumption is central to the model of Keynes' General Theory, which is often considered to be the origin of macroeconomics. Keynes specified a simple linear consumption function in which consumption is a positive function of disposable income. Though consumption depends on disposable income, there is a part of consumption which does not depend on disposable income and this is called autonomous consumption. The portion of the consumption function which depends on disposable income is called induced consumption and the marginal propensity to consume shows how much consumption will change when income changes. Three points can be deduced from the consumption function stipulated by Keynes. First, the value of marginal propensity to consume is constant and less than one, thus consumer increases their consumption as their income increases, but not as much as the increase in their income. Secondly, the average propensity to consume which is the ratio of total consumption to total income falls as the level of income increases. For Keynes, individuals consider saving as a luxury and this explains why the rich save a higher proportion of their income than the poor. The third deduction from Keynes' consumption postulated that current consumption depends only on current income. Consumption models built on the initial work by Keynes are as follows: Irving Fisher's Inter-temporal choice model; The Life Cycle Hypothesis (LCH) developed by Modigliani and Brunberg (1954); and the Permanent Income Hypothesis (PIH) by Friedman (1957).

The Engel curve

The Engel curve describes the relationship between household expenditure and income. Engel (1857) initiated the studies on household food expenditure survey and found that food expenditure was an increasing function of income and family size, but the food budget shares declined with income. This finding led to the formulation of the Engel's law which states that "the poorer a family is, the larger the budget share it spends on nourishment" (Engel, 1857, pp. 28-29). Thus, the Engel's law can be used to evaluate the

general welfare of households and in particular the conditions of poor households.

According to Engel (1857), food expenditure is an essential expenditure which dominates low income household expenditure patterns; a fall in households' income thus, tends to crowd out expenditure on other non-essential goods. However, when households' incomes increase, a smaller percentage of it is spent on food while a large portion goes into non-food items. Similarly, the finding by Engel (1857) shows that the proportion of income allocated to food is directly related to household size, where larger households spend a higher share of their income on food than smaller households.

Analytical approach

Some food expenditure studies (Yemer, 2011) estimate a Tobit model with the explanation that a significant number of households record zero values. Thus, in the case where there are no zero values the ordinary least squares method (OLS) is appropriate. Also, most of these studies (Ayo et al., 2012; Begum et al., 2010; Umeh and Asogwa, 2012) estimate a single equation with income as one of the main explanatory variables. The problem with the single equation estimation is that income is assumed to be exogenous, yet income is not truly exogenous; while income determines food expenditure, it is determined itself by other variables including food expenditure. This means that estimating a single equation and making income exogenous results in simultaneous bias (Koutsioyannis, 1977; Gujarati, 2004). The right model should be a simultaneous equation involving two equations, each for food expenditure and income. The most appropriate estimator for a simultaneous equation model involving two equations is that of the two stage least squares (2SLS). Lastly, even though in practice, income is equivalent to welfare as defined by the Ghana Statistical Service (2007), in principle, welfare is broader than income because the former includes other indicators of wellbeing in addition to income. Besides, people tend to underestimate their incomes and so the use of expenditure as a measure of welfare as used in the GLSS is preferred.

Simultaneous equations: Two stage least square (2SLS)

Gujarati (2004) shows that when we estimate a single equation by OLS, but the equation has one or more explanatory variable(s) that is/are endogenous, it results in simultaneous equation bias. The right approach is to develop a simultaneous equation system and estimate it by a two stage or three stage least squares depending on the number of endogenous variables. Given the following structural equations:

$$y_1 = b_{12}y_2 + \gamma_{11}x_1 + \gamma_{12}x_2 + \cdots + \gamma_{1i}x_i + u_1 \quad (1)$$

$$y_2 = b_{22}y_1 + \gamma_{21}x_1 + \gamma_{22}x_2 + \cdots + \gamma_{2i}x_i + u_2 \quad (2)$$

where y_1 and y_2 are endogenous variables; x_i are predetermined variables; b_{12} and b_{22} are coefficients of the endogenous variables and γ_{ji}'s are coefficients of predetermined variables; u_1 and u_2 are the random terms with zero mean, constant variance and zero covariance, but non-zero covariance between the y_i and the u_i.

The reduced form of the structural model is obtained by solving the structural equations simultaneously as follows:

Substituting Equation 2 into 1 we obtain:

$$y_1 = b_{12}(b_{22}y_1 + \gamma_{21}x_1 + \gamma_{22}x_2 + \cdots + \gamma_{2i}x_i + u_2) + \gamma_{11}x_1 + \gamma_{12}x_2 + \cdots + \gamma_{1i}x_i + u_1$$

$$y_1 = \frac{1}{(1-b_{12}b_{22})}[b_{12}(\gamma_{21}x_1 + \gamma_{22}x_2 + \cdots + \gamma_{2i}x_i + u_2) + \gamma_{11}x_1 + \gamma_{12}x_2 + \cdots + \gamma_{1i}x_i + u_1] \quad (3)$$

Thus;

$$y_1 = \pi_{11}x_1 + \pi_{12}x_2 + \pi_{13}x_3 + \cdots + \pi_{1i}x_i + v_1$$

where

$$\pi_{11} = \frac{b_{12}\gamma_{21}+\gamma_{11}}{((1-b_{12}b_{22}))}; \pi_{12} = \frac{b_{12}\gamma_{22}+\gamma_{12}}{((1-b_{12}b_{22}))}; \pi_{1i} = \frac{b_{12}\gamma_{2i}+\gamma_{1i}}{((1-b_{12}b_{22}))} \text{ and } v_1 = \frac{b_{12}u_1}{(1-b_{12}b_{22})}$$

Similarly, substituting Equation 1 into 2 we obtain:

$$y_2 = b_{22}(b_{12}y_2 + \gamma_{11}x_1 + \gamma_{12}x_2 + \cdots + \gamma_{1i}x_i + u_1) + \gamma_{21}x_1 + \gamma_{22}x_2 + \cdots + \gamma_{2i}x_i + u_2$$

$$y_2 = \frac{1}{(1-b_{12}b_{22})}[b_{22}(\gamma_{11}x_1 + \gamma_{12}x_2 + \cdots + \gamma_{1i}x_i + u_1) + \gamma_{21}x_1 + \gamma_{22}x_2 + \cdots + \gamma_{2i}x_i + u_2]$$

The reduced form is represented as;

$$y_2 = \pi_{21}x_1 + \pi_{22}x_2 + \pi_{23}x_3 + \cdots + \pi_{2i}x_i + v_2$$

where

$$\pi_{21} = \frac{b_{22}\gamma_{11}+\gamma_{21}}{((1-b_{12}b_{22}))}; \quad \pi_{12} = \frac{b_{22}\gamma_{12}+\gamma_{22}}{((1-b_{12}b_{22}))}; \quad \ldots; \pi_{1i} = \frac{b_{22}\gamma_{1i}+\gamma_{2i}}{((1-b_{12}b_{22}))} \text{ and } v_2 = \frac{b_{22}u_2}{(1-b_{12}b_{22})}$$

Thus, the reduced forms of the structural model for the two endogenous variables are:

$$y_1 = \pi_{11}x_1 + \pi_{12}x_2 + \pi_{13}x_3 + \cdots + \pi_{1i}x_i + v_1 \quad (4)$$

$$y_2 = \pi_{21}x_1 + \pi_{22}x_2 + \pi_{23}x_3 + \cdots + \pi_{2i}x_i + v_2 \quad (5)$$

It can be observed that y_i are correlated with u_i because from the reduced-form y_i are correlated with v_i. Hence we cannot obtain consistent estimates of the coefficients of the structural equations if we estimate them by OLS. The equations need to be estimated by the two-stage-least squares (2SLS).

Using the two stage least squares method to estimate the simultaneous equations system, we need to first apply the ordinary least squares to the reduced form Equations (4) and (5) to obtain estimates of the $\hat{\pi}$'s. Using the reduced form coefficients we obtain a set of computed values for the endogenous variables $\hat{y}_1$ and $\hat{y}_2$.

In the second stage the estimated endogenous variables $\hat{y}_1$ and $\hat{y}_2$ are substituted into the structural Equations (1) and (2) to obtain the transformed equation as:

$$y_1 = b_{12}\hat{y}_2 + \gamma_{11}x_1 + \gamma_{12}x_2 + \cdots + \gamma_{1i}x_i + u_1^* \quad (6)$$

$$y_2 = b_{22}\hat{y}_1 + \gamma_{21}x_1 + \gamma_{22}x_2 + \cdots + \gamma_{2i}x_i + u_2^* \quad (7)$$

where

$$u_1^* = u_1 + b_{22}v_2; \; u_2^* = u_2 + b_{12}v_1$$

Solving the transformed structural Equations (6) and (7) by using the ordinary least squares we obtained the 2SLS estimates of the structural parameters. However, to be able to estimate the model by 2SLS it must satisfy the order and rank conditions. These are well spelt out in Koutsoyiannis (1977) and Gujarati (2004).

Table 1. Summary definition of variables.

Variable	Description
Sex of household head	Dummy variable; 1 if head is male and 0 if female
Age of household head	No of years(in logarithm)
Age squared	No of years squared (in logarithm)
Education of household head	No of years of formal education
Marriage of household head	Dummy; 1 if head is married, 0 otherwise
Household size	No of members in the household(in logarithm)
Land	Dummy; 1 if household own land; 0 if otherwise
vehicle	Dummy; 1 if household own a commercial vehicle, 0 if otherwise
Durable assets	Total value in millions of Cedis of household durable assets(in logarithms)
Locality	Dummy; 1 if household lives in urban center and 0 if in rural area
Coastal zone (Coastal)	0 if household lives in forest zone and 1 if otherwise
Savannah zone (Savannah)	0 if household lives in forest zone and 1 if otherwise
Regional distance	Distance in kilometres from Accra (the national capital) to the capital of the region in which a household lives
Welfare	Household total nominal expenditure divided by the product of Accra price index and the national equivalence scale
Food Expenditure	Percentage of household expenditure on food
Poverty status	Categorical; 0 if welfare is below the lower poverty line; 1 if welfare is between the lower and upper poverty lines; 2 if welfare is above the upper poverty line

10,000 (old) Cedis = (New) Gh ¢ 1; Exchange rate at the time was $1= Gh ¢1.

Table 2. Descriptive statistics of continuous variables used in the model.

Variable	No.	Minimum	Maximum	Mean	Std. deviation
Age	3941	15	99	45.53	15.820
Household size	3941	1	27	4.18	2.823
Educational level	3941	0	16	6.18	5.326
Durables	3941	0	127000000	971743.59	4421766.889
Food expenditure	3941	00.369117	95.79412	56.9049777	16.06479846
Welfare	3941	75020.39	81700000.00	2209776.84	2499793.64

Empirical model

The empirical model for this present study consists of two main equations, namely, the food expenditure and welfare equations as specified below. Note that they satisfy both the order and rank conditions.

$$Pfexp = \alpha\_0 + \alpha\_1\, Sexhead + \alpha\_2\, Agehead + \alpha\_3\, Agesqd + \alpha\_4\, Educhead + \alpha\_5\, Marhead +$$

$$\alpha_6 HHsize + \alpha_7 Locality + \alpha_8 Coastal + \alpha_9 Savanna + \alpha_{10} Region + \alpha_{11} Welfare + u_1$$

(Food Expenditure)

$$Welfare = \beta\_0 + \beta\_1\, Sexhead + \beta\_2\, Agehead + \beta\_3\, Agesqd + \beta\_4\, Educhead + \beta\_5\, HHsize + \langle\!\langle \beta \rangle\!\rangle\_6\, Land + \beta\_7\, Vehicle + \beta\_8\, Durables + \beta\_9\, Locality + \beta\_10\, Region + \langle\!\langle \beta \rangle\!\rangle\_11\, Pfexp + u\_2$$

(Welfare)

The variables are defined in Table 1.

Data and descriptive statistics of continuous variables

The study uses data from the fifth round of the Ghana Living Standards Survey (GSS, 2008). From Table 2, the average age of a household head was 45 years while the average size of a household was 4. This is exactly the national average. Generally, rural households are larger than urban households. In terms of ecological zone, rural savannah recorded the highest average household size (5.4) in 2007, followed by rural forest (4.1), and the rural coastal (GSS, 2008). GSS (2008) noted that a combination of

Table 3. Frequency Distribution of Percentage of food expenditure.

Class interval	Frequency	Percentage
0-19	56	1.4
20-39	517	13.1
40-59	1549	39.3
60-79	1551	39.4
80-99	268	6.8
Total	3941	100.0

Table 4. Two stage and OLS estimation results of food expenditure equation.

Variable	2SLS		OLS	
	Coefficient	Standard Error	Coefficient	Standard Error
Constant	88.0407	3.1915	75.3627	3.1457
Sexhead	-1.4569**	0.5270	1.5166***	1.4993
Agehead	-0.6062***	0.0773	-0.5299***	0.0789
Agesqd	6.2095***	0.7410	5.6218***	0.7561
Educhead	-0.4701***	0.0542	-0.8568***	0.0487
Marriage Head	1.0464***	0.0030	1.0464***	0.0030
Household size	-2.0748***	0.1511	-0.5128***	0.1124
Locality	-5.7584***	0.6043	-11.4464***	0.4826
Coastal	-10.1243***	0.6127	-8.8675***	0.6202
Savannah	18.7627***	1.2422	14.8633***	1.2414
Region	0.0689***	0.0129	0.0463***	0.0131
Welfare	-4.5086***	0.3413	0.1944***	0.1420

Adjusted R-Squared = 0.99.

factors determines the household size in Ghana. The main ones are a desire, especially on the part of traditional families, to have large families. In the case of urban families, it is the extended family system, which compels them to take care of dependants, other than their immediate family members.

The average number of years that a household head spent in school was 6. This means that on average household heads completed primary six which takes a minimum of six years. The national statistics (as of 2008) was as follows: about 31% of all adults had never been to school; less than 17.1% attended school but did not obtain any qualification; 39% had MSLCE/BECE/VSCE certificate as their highest qualification, while a small percentage of 13.6 had secondary or higher qualification (GSS, 2008). The average wealth of durable assets (such as television set, bicycles and sewing machines) was GH¢997.00 an equivalent of about $500.00 today. Also the average value of welfare was GH¢220.97 an equivalent of about $110.00 in current terms. Lastly, the mean percentage of income spent on food was 65%. However, from Table 3, the majority of the respondents (78.7%) spent between 40 and 79% of their incomes on food.

Estimation results of the 2SLS and OLS compared

From Tables 4 and 5 we notice some differences in the 2SLS and the OLS estimation results. For instance, in Table 4 while the welfare variable is negative in the 2SLS it is positive in the OLS. Also, while sexhead has a negative coefficient in the 2SLS, it is positive in the OLS. Similarly, in Table 5, region has a negative coefficient in the 2SLS but is positive in the OLS. Obviously, the true relationship between food expenditure and welfare would have eluded us if we had used OLS estimator instead of the 2SLS.

The determinants of food expenditure

All the variables that were suspected to influence household food expenditure were significant (Table 4), most of them maintaining their expected signs. The Adjusted R-squared of 0.99 shows that the explanatory variables were able to explain 99% of the variation in food expenditure. Thus, the model was good. Also, the negative significant coefficient of the welfare variable means that an increase in household welfare leads to a reduction in the percentage of household budget on food expenditure. This is consistent with the Engel theory (1885) that households reduce their budget share of food as they become richer. At this stage, it is important a distinction is drawn between the findings of studies that used absolute food expenditure values (Begum et al., 2010; Yimer, 2011; Akpan et al., 2013) and those which used the budget share of food expenditure like our present study (Umeh and Asogwa, 2012).

Generally, in the former studies, income had a positive effect on the level of consumption, though in some instances, the relationship was negative. For instance, in Yimer (2011) study in Ethiopa, while income had a positive effect on the consumption of teff, it had a negative effect on maize consumption. Similarly, in Akpan et al. (2013) study, while workers' salaries had positive effects on food consumption in Southern Nigeria, other sources of income such as farm and non-farm income as well as income from other family

Table 5. Two stage and OLS estimation results of welfare equation.

Variable	2SLS		OLS	
	Coefficient	Standard error	Coefficient	Standard error
Constant	9.7659	0.5883	3.6695	0.3407
Sexhead	-0.3458***	0.0512	-0.5681***	0.0490
Agehead	-0.0806***	0.0078	-0.0306***	0.0069
Agesqd	0.7835***	0.0753	0.2698***	0.0645
Educhead	0.0246***	0.0068	0.0786***	0.0054
Household size	-0.3784***	0.0114	-0.3505***	0.0114
Vehicle ownership	0.3148***	0.0444	0.3609***	0.0451
Land ownership	0.0169***	0.0019	0.0126***	0.0019
Durable assets	0.0002	0.0003	0.0029***	0.0017
Locality	0.7420***	0.0604	1.2299***	0.0474
Region	-0.0041***	0.0011	0.0007***	0.0011
Food Expenditure	-0.0632***	0.0051	-0.0025***	0.0016

Adjusted R-Squared = 0.99.

members had negative effects on food consumption. However, in the study by Umeh and Asogwa (2012) where the dependent variable was food consumption budget, the variable was consistently negatively related to household income, consistent with our study and for that matter the Engel theory.

Other variables like education and household size appeared to go the same direction as income depending on whether the study used absolute or percentage share of food expenditure (Yimer, 2011; Meng et al., 2012; and Akpan et al., 2013). To sum up, the findings in Table 4 indicate that households which spent greater percentages of their incomes on food were as follows: female headed households; households headed by the aged, households whose heads had little or no formal education; households whose heads were married; smaller households; rural households; households in the forest belt as opposed to those in the coastal zone; households in the savannah belt; households living farther from the nation's capital; and poorer households. As indicated earlier most of these findings are consistent with that of the above mentioned studies.

The determinants of welfare

The welfare model was also good in explaining the variations in household welfare, considering the 99% Adjusted R-Squared value reported in Table 5. It can also be observed that all the variables were significant, except durable assets. The negative sign of the food expenditure variable shows that as the percentage of a household income spent on food increased, the welfare of that household reduced. In general, in this study, welfare was greater for the following households: female headed households; households headed by the aged, households whose heads had formal education, smaller households, households who owned assets such as land and commercial vehicles; households living in the urban centres, as well as those living closer to the nation's capital. These findings are also consistent with that of similar studies (Gibson and Rozelle, 2003; Datt and Jollife, 2005; Coulombe, 2008).

RESULTS AND DISCUSSION

As noted earlier, many theoretical and empirical studies have established a negative relationship between food expenditure and income, implying that the higher the income of a household the lower the percentage of the income spent on food, and the lower the income the higher the proportion allocated to food consumption. In this present study, the negative relationship between households' welfare and their food budget share implies that poorer households spend a greater percentage of their incomes on food than richer households. In line with Engel (1885), Umeh and Asogwa (2012) noted that poorer households spend large percentage of their incomes on necessities, including food. However, as their incomes increase they divert more of their incomes to buying higher quality goods and services, thereby reducing the proportion that goes into food. It should be noted that the emphasis is on the proportion of income that goes into food and not the absolute income. Obviously, the absolute income spent on food by a rich household is greater than that of a poorer household, *ceteris paribus*, but in terms of the percentage of income spent on food, that of the latter is likely to be higher. This is validated further by Figure 1a and b. It can be observed that in terms of the percentage of food expenditure, poorer households recorded higher, but in terms of food expenditure in absolute terms they recorded lower than their richer counterparts. The implications of this for research is what we mentioned earlier that a distinction should be drawn between studies that use the absolute food expenditure value and those that use the budget share of food expenditure as the dependent variable. While the former generally establish a positive causal relationship between income and food expenditure, the latter, like our present study establish a negative relationship.

The negative sign of food expenditure in the welfare equation also confirms the fact that higher food expenditure makes households poorer *ceteris paribus.* This is understandable, considering the fact that a

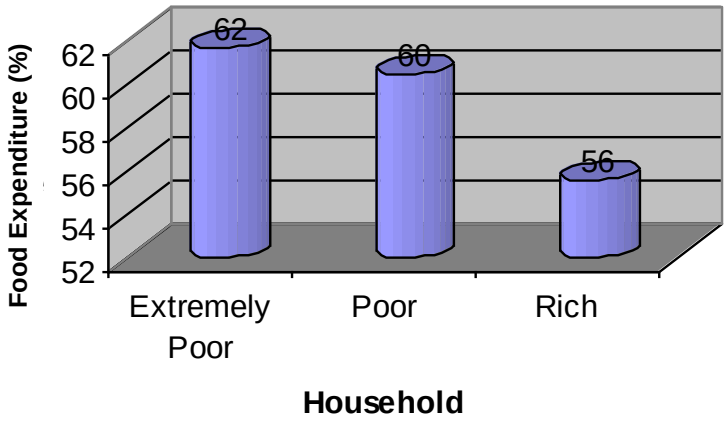

Figure 1a. Poverty status of households and percentage food expenditure.

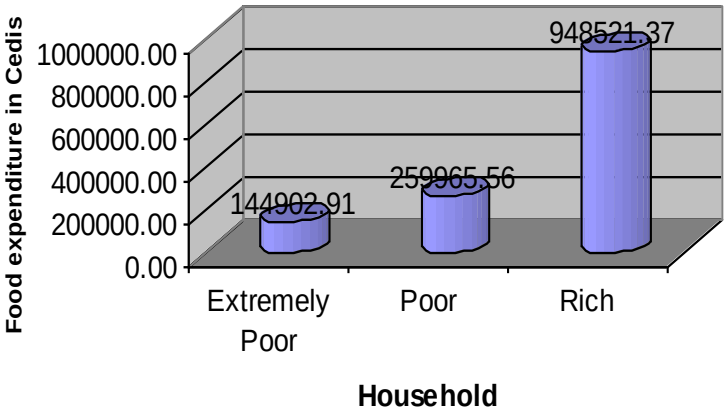

Figure 1b. Poverty status of households and food expenditure.

household spending a greater percentage of its income on food means that it has a smaller percentage left for other goods and services which are vital for its welfare. In Umeh and Asogwa (2012) study, while per capita income negatively influenced the share of food expenditure, therelationship between per capita income and the share of non-food expenditure was positive, confirming the fact that as a household's income rises it increases its share of expenditure on housing, clothing, education and health, among others, to improve upon its standard of living.

A close study of the results from the two equations reveals two main categories of households; (i) households who spent a greater percentage of their income on food but were richer (female headed households, households headed by the old and smaller households) and (ii) households who were poorer and also spent a smaller percentage of their income on food (male-headed households, households headed by the relatively young, larger households, rural households and households living farther away from the nation's capital). The latter group must be targeted for support. Other households that need support are households living in the savannah zone as well as households that had no assets such as land and commercial vehicles.

Female-headed households emerging as richer than male-headed households were contrary to our *a priori* expectations. We also observe in Figure 2a and b that in terms of both the food budget shares and the absolute food expenditure, they recorded higher figures than their male counterparts. Thus, the finding of the present study does not support the issue of feminization of poverty as found by Rodriguez (2000). Following the Beijing Conference on gender inequality a lot of support from both governmental and non-governmental organizations has been given to women in Ghana. Perhaps, this explains why female-headed households are now doing better in terms of welfare, than their male-headed counterparts in Ghana. While the support for women and for that matter, female-headed households should continue, their male counterparts should also be supported since by the findings of this study, they are not

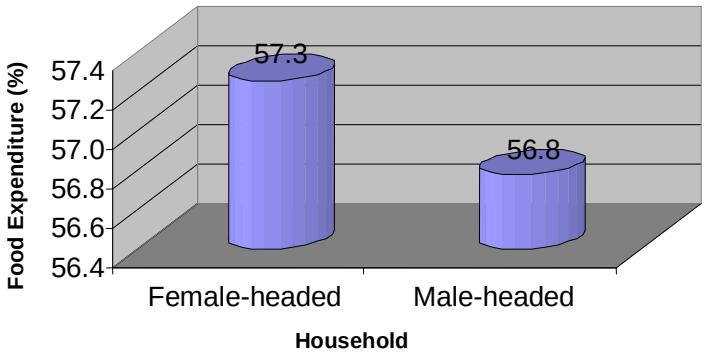

Figure 2a. Sex of Household Head and Percentage of Food Expenditure

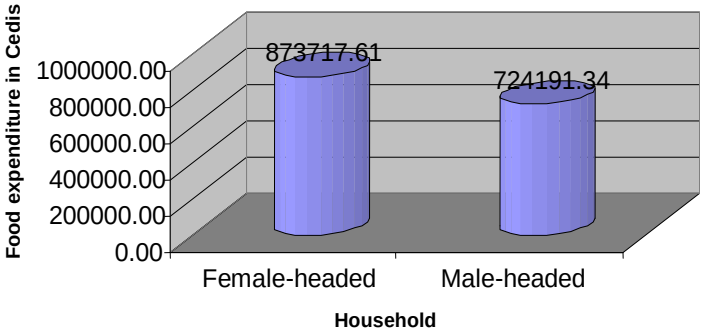

Figure 2b. Sex of Household Head and Food Expenditure.

only poorer but have smaller budget share of food expenditure.

An equally important group that needs support, as per these findings, is large households. As argued by Umeh and Asogwa (2012), the budget share of food expenditure for large households is smaller because they are likely to spend more on non-food items such as education and health. Recall that in the welfare model, larger households were poorer than smaller households, which means that the non-food expenditure here is not a sign of affluence but a necessity. Thus, by virtue of the household size being large, they are forced to cut down the budget share of food expenditure in order to take their children to school or seek medical attention. This is different from an affluent household spending more on luxurious or high quality products because they have a significant increase in their income. For instance, from Figures 3a, b, 4a and b we notice that even though the budget share of food expenditure for households headed by illiterates and households in the rural areas were higher, in terms of the absolute food expenditure, their figures were lower than households headed by literates as well as those in the urban centres, respectively.

It is also important that the gap, in terms of economic resources and opportunities, is closed between the nation's capital and those farther away. This calls for equitable distribution of the national cake and the implementation to the letter of the decentralization policy that is being pursued. Again, Figures 5a, b, 6a and b reveal that while the savannah zone and the three northern regions in general, recorded high percentages of food expenditure, the absolute food expenditure figures for these regions were lower than their counterparts in the south. For a long time, the economic disparity between the south and the north of the country has caught the attention of many analysts and policy makers. Consistently, the Ghana Living Standards Survey data have established that in addition to the central region, the three northern regions are the poorest, of the ten regions of Ghana (GSS, 2007). Food insecurity is associated with poverty or lack of wealth (WFP, 2012). According to WFP(2012), the Upper East has the highest proportion of households who are food insecure (28%), followed by Upper West (16%) and the Northern region (10%).

Conclusion

The main objective of this study was to investigate the determinants of household food expenditure and its effects on welfare. Specifically, the study sought to find out the extent to which household budget share of food

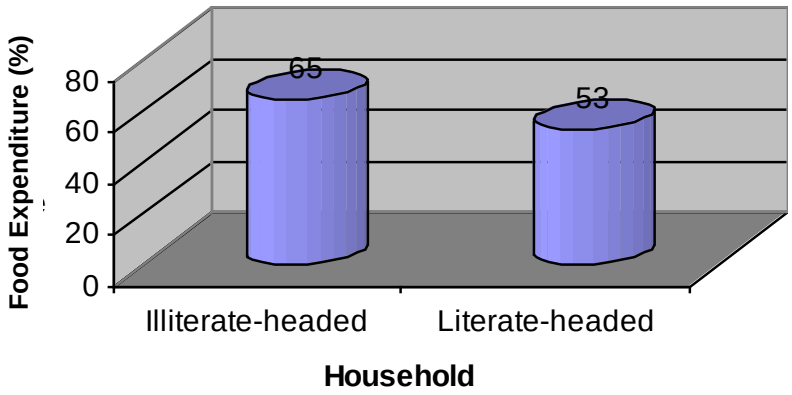

Figure 3a. Household heads' educational background and percentage of food expenditure.

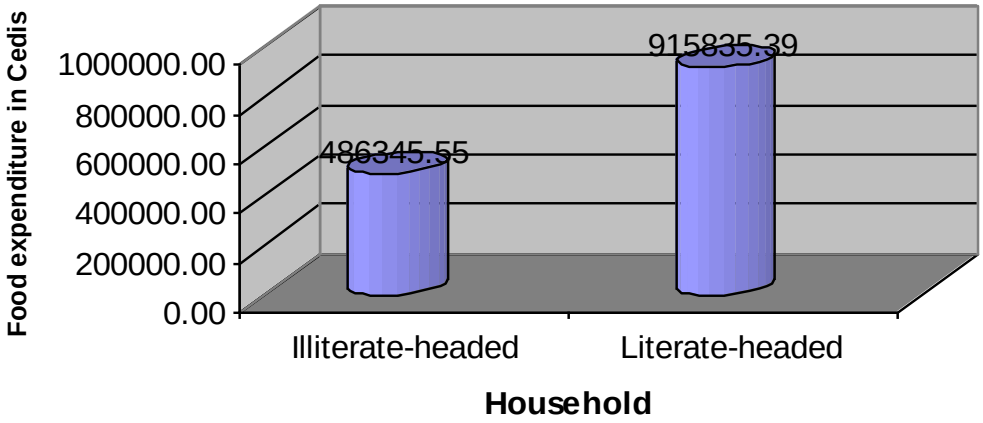

Figure 3b. Household heads' educational background and food expenditure.

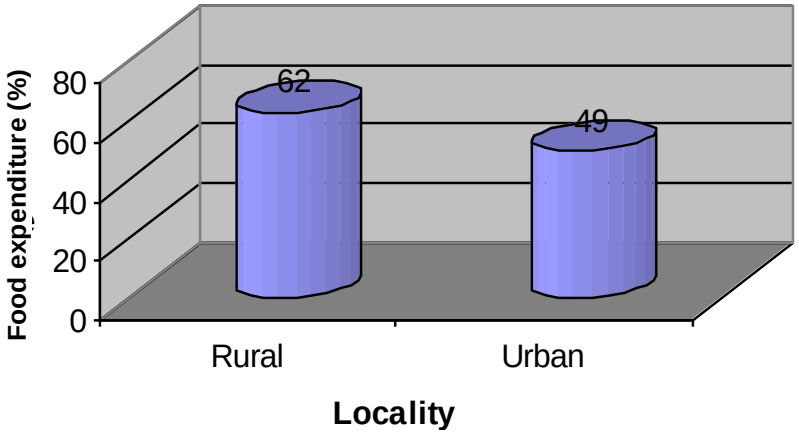

Figure 4a. Locality and percentage of food expenditure.

expenditure (percentage of income spent on food) determined their welfare and *vice versa*. As a result of the simultaneity between food expenditure and welfare, we estimated a simultaneous equations model by the two-stage least squares method. The negative and significant coefficients of the food expenditure and welfare variables

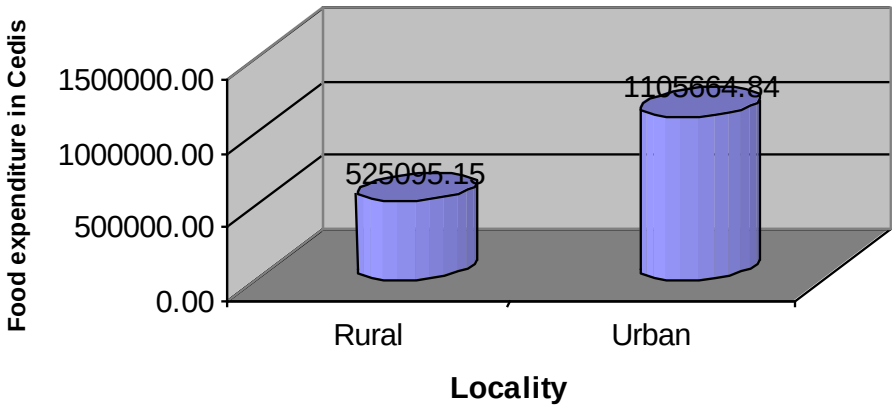

Figure 4b. Locality and Food Expenditure.

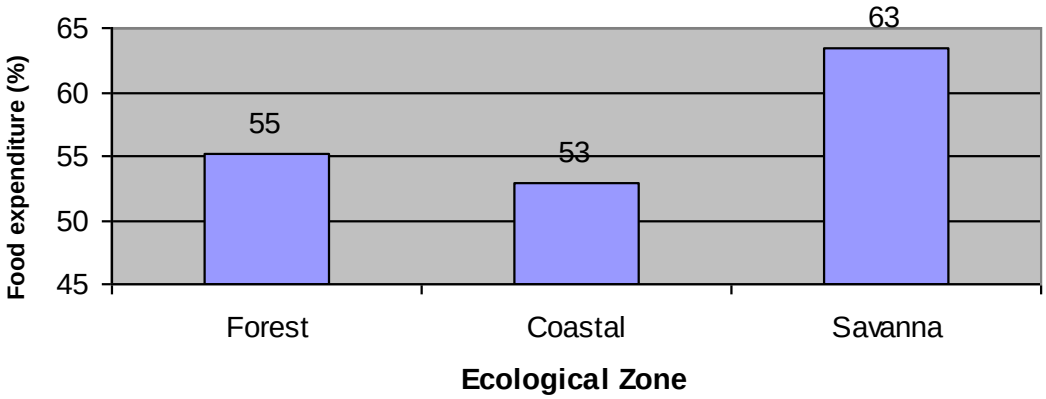

Figure 5a. Ecological zone and percentage of food expenditure.

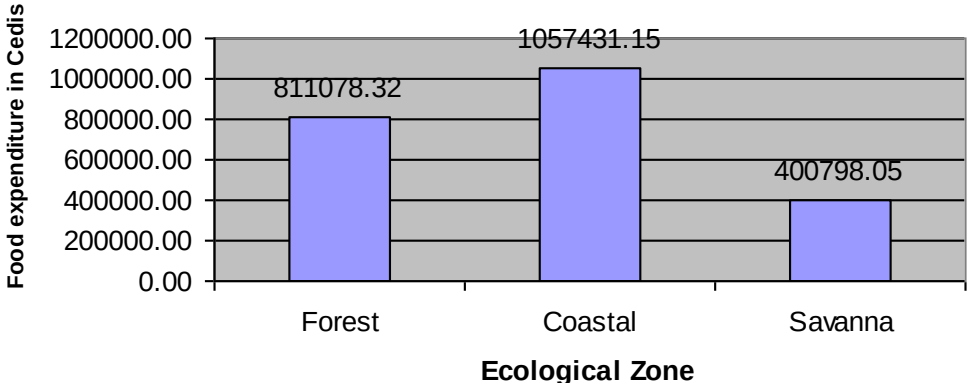

Figure 5b. Ecological zone and food expenditure.

confirm the theoretical and empirical evidence that households reduce the percentages of their incomes allocated to food expenditure as they become rich.

The relatively rich households were as follows: female headed households; households headed by the old; households headed by literates; smaller households; households who owned land, commercial vehicles and other durable assets; and households in the urban centres or closer to the nation's capital. Generally, apart from female-headed households and households headed

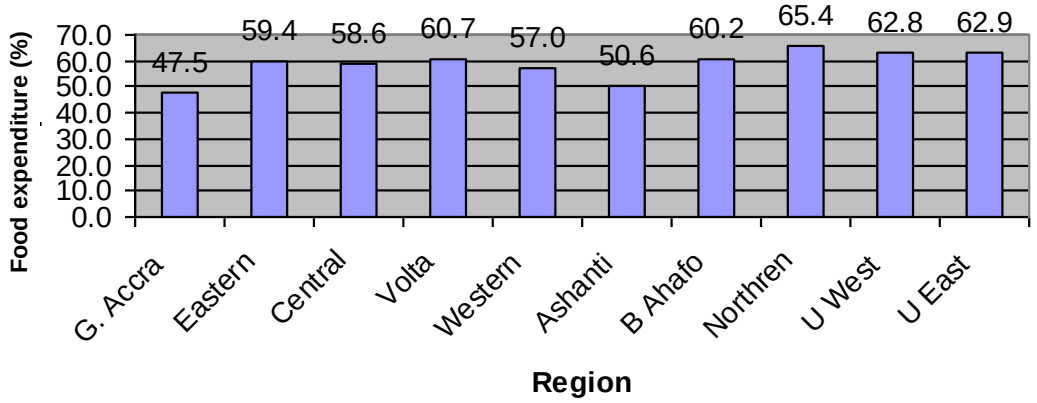

Figure 6a. Region and percentage of food expenditure.

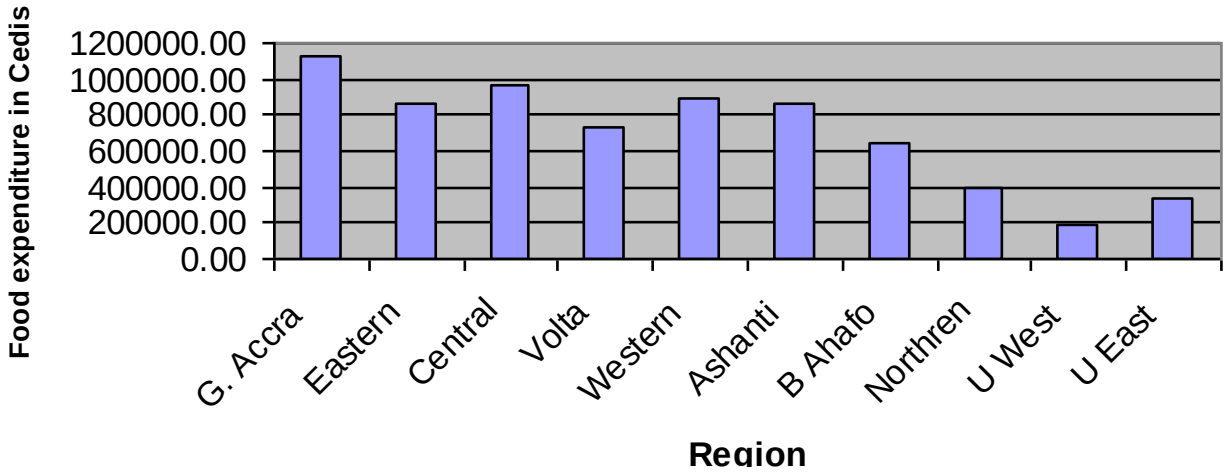

Figure 6b. Region and food expenditure.

by the old, these households spent smaller percentages of their incomes on food, but their absolute food expenditure figures exceeded their relatively poor counterparts such as male-headed households, households headed by the relatively young, larger households, rural households and households farther from the nation's capital, including those in the savannah belt. This latter group must have priority in terms of support.

The contribution of this study to existing literature is three-fold; first while many studies used absolute food expenditure as the dependent variable, we have used the budget share of food expenditure (percentage of household income spent on food). Second, in place of income, we have used welfare, which in principle is broader in scope and much more realistic than income which respondents tend to underestimate. Finally, instead of a single equation, we have estimated a simultaneous equation system; because food expenditure and welfare are both endogenous.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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