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Short Communication

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A New Insight into Cold Stress In Poultry Production

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Since about 1950, numerous examples of extreme climate events have been recorded. These include not only the increase in temperature which has caused frequent and intense heat waves, melting ice caps, and high sea level, but also an extreme low winter temperature along with higher precipitation in different regions in the world. Further changes in the climate system are predicted to increase over the course of the 21st century.[1] Since growing animals are vulnerable to extreme temperature, climate changes become an important critical constraint to several species in the world.[1,2] In poultry production, while heat stress has been a rising concern for producers and scientists, cold stress has also caused economic loss worldwide. In China, winter conditions and low temperature caused almost 20 million poultry deaths and an economic loss of 100 million (Chinese currency) in 2008.[3] Early research in poultry exposed to acute cold stress has shown a clear suppression in development, survival and egg production.[4-6]

At the molecular level, an acute hypothermal condition significantly up-regulated gene expression of hepatic leptin and muscle UCP in 5-wk-old broilers.[7] Exposure at 4 ˚C for 24 hours resulted in changes in some genes involved in lipid metabolism in broilers pituitary. This suggests that cold stress can affect lipid metabolism.[3] At younger age (7-14 days old), although a chronic cold stress (20 ˚C) did not affect body weights and feed intake, it significantly increased chick body heat production and *avUCP* gene expression in the leg muscle.[8] Birds exposed to cold stress had severely injured liver and increased gene expression of AMPKα–PPARα pathway.[9] Moreover, there is clear evidence that cold stress affects thyroid hormones (T3 and T4) which play a key role in energy expenditure and body temperature homeostasis.[8,10] Yet, the effect of cold exposure on growth performance is still controversial. Indeed, Baarendse and colleagues[11] observed that moderate cold exposure (28 ˚C, reducing 1 ˚C every day in five-day period) during the early post-hatching period caused long-term negative effects on chicken growth performance. However, Shinder and colleagues reported that acute cold exposure at late embyogenesis improved growth performance.[10,12] These discrepancies might be due to several factors including experimental conditions (environmental temperature, age, chicken strain, and/or exposure duration). We recently investigated the effect of Chronic Mild Cold Conditioning (CMCC) and its underlying molecular mechanisms on growth performances in broilers. Our data show that CMCC improved the growth performance of chicks during the first week post-hatch and their later lives in terms of body weight gain and Feed Conversion Ratio (FCR).[13] Plasma cholesterol and creatine kinase (CK) levels increased indicating a potential role of CK in maintaining high ATP turnover in a hypothermal condition.[14] Hypothalamic orexigenic neuropeptide Y (NPY) and anorexigenic cocaine and amphetamine regulated transcript (CART) gene expression were significantly down-regulated in the brain of cold group which may explain the reduction of feed intake in CMCC compared to the control group. CMCC also modulated the hepatic expression of lipogenic genes, which implies the inhibition of fatty acid synthesis in cold stress chicks.[13] Moreover, CMCC enhanced muscle fatty acid β-oxidation through affecting the gene and protein expression of carnitine palmitoyl transferase-1 (CPT-1) and phosphorylated mTOR.[16,17]

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In summary, the CMCC used in our study could improve later growth performance of young chicks (body weight gain and FCR). This is new evidence that gives us a broader view of how young birds can adapt to and prepare for changes in their environment. In addition, gene expression analyses provide insight into the roles of the AMPK-mTOR pathway in cold acclimation; thus further studies are needed to understand the regulation of these genes for better management *via* genetic selection and/or nutritional strategies to improve cold tolerance and feed efficiency.

CONFLICTS OF INTEREST: None.

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Research

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Physicochemical and Organoleptic Characteristics of Dehydrated Apricots under Different Drying Conditions

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ABSTRACT

The present study was carried out to investigate the effect of different drying methods on the physicochemical composition and organoleptic characteristics of dehydrated apricot fruits. The fresh apricot was dehydrated in open sun and in moveable solar drier developed by Pakistan Council of Scientific and Industrial Research (PCSIR) Skardu. The chemical composition showed that the fresh apricots contained moisture 83.3%, ash 0.72%, crude fat 0.03%, crude Protein 0.9%, crude fiber 1.02% and carbohydrates 14.03%. The moveable solar drier and using open sun drying substantially decreased moisture content to 14.61% and 15.7% respectively. Proportions of other components were increased, which include ash (3.51% and 3.43%), crude fat (1.99% and 1.82%), crude protein (1.0% and 0.97%), crude fiber (2.98% and 2.95%) and carbohydrates (75.91% and 75.13%). Organoleptic characteristics of open sun dried apricot has a little negative effect on over all acceptability when compared to moveable solar dehydrated apricot, however the open sun dried method was declared acceptable by the panel of judges for color, taste and overall acceptability.

KEYWORDS: Apricot; Dehydration; Sun drying; Moveable solar drier.

ABBREVIATIONS: PCSIR: Pakistan Council of Scientific and Industrial Research; GB: Gilgit-Baltistan; FTC: Food Technology Center; PSF: Pakistan Science Foundation.

INTRODUCTION

Gilgit-Baltistan (GB) is the most important part of the country extends over an area of 27188 sq miles. Administratively it is distributed among 10 Districts (Gilgit, Skardu, Diamer, Astore, Ghagchae, Ghizer, Hunza, Nagar, Shigar and Kharmang) with a population of 2 million. The main issue of Gilgit-Baltistan is food insecurity as cultivated lands are less than one kanal per capita.[1,2] The people of GB totally depend on wheat supplied through Government on subsidized rates form Punjab.[3-5] Apricot (*Prunus armeniaca L.*) is one of the most important, attractive, delicious, highly nutritious and major fruits of Gilgit-Baltistan. The fruit tree grows from plain to altitude of 3000 meters.[6] The fruit is having a distinct pleasant aroma and is used for preparing many products including jam and nectar. The dried fruit is available in the market round the year, while the fresh fruit comes in the market by the end of May to September.[7] Due to lack of processing, preservation, testing, transportation, communication and research large amount of fruits and vegetables are wasted and do not reach in distant markets because of their perishability.[8] To overcome the food security issues of Gilgit-Baltistan and to cope the tremendously increasing demand of food locally without bringing more land under cultivation. Dehydration, processing and preservation of fruits through trainings to farming community are milestone.[9,10] The present work was thus under taken to evaluate the chemical and organoleptic

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characteristics of dehydrated apricot and to compare their quality on the basis of nutritional significance under different drying methods used in Gilgit-Baltistan.[11]

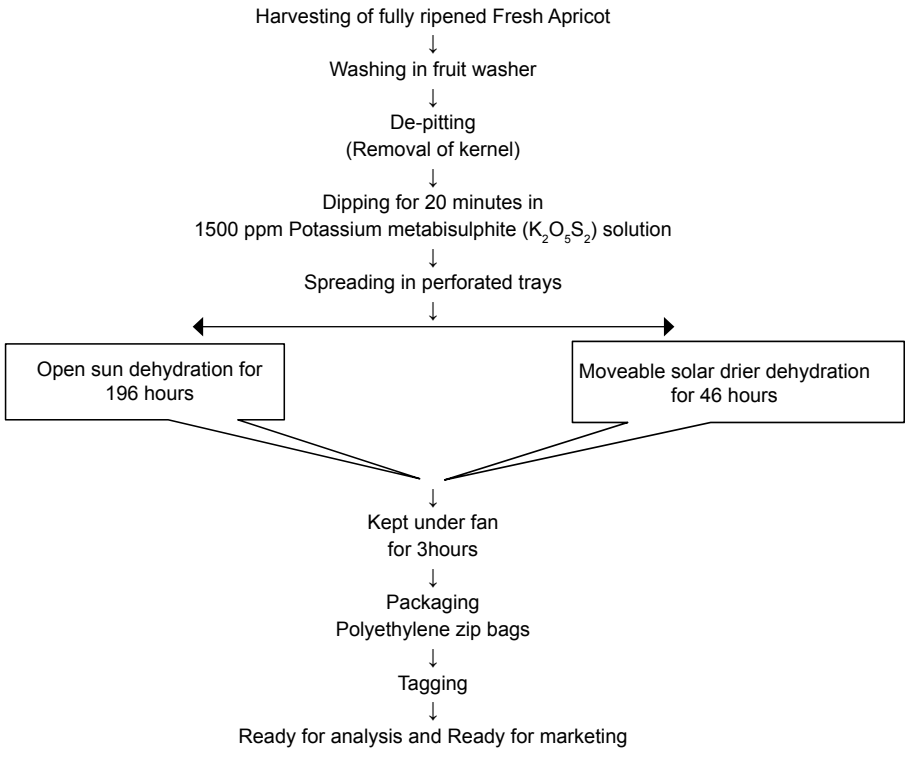
MATERIALS AND METHODS

Dehydration of Fruits

Proper healthy and mature Apricot (Halman variety) fruits were selected for this study. The fruits were washed with deionized water and dipped in already prepared 1500 ppm potassium metabisulphite solution[12-14] for 20 minutes. The fruits were then kept in pre-washed stainless steel perforated trays. The trays were put in moveable solar drier and in open sun on the roof of PCSIR processing hall. The moveable solar drier moved according the direction of sun 8:00 am and 3:00 pm.[15,16] The moveable solar drier temperature reached to 70-75 °C maximum and the open sun maximum temperature was noted up to 20-27 °C during the month of July. The apricot dehydrated in moveable solar drier during 46 hours (approximate 2 days) while apricot dehydrated in open sun during 196 hours (8 days and 4 hours). The trays collected from moveable solar drier and open sun were packed in polyethylene zip bags with proper tags for further physicochemical and organoleptic evaluation.[3,17] (See Flowsheet diagram)

Physicochemical Analysis

Moisture, total ash, crude fat, crude protein, crude fiber and carbohydrates were determined according to the Association of Analytical Communities (AOAC) methods. Crude protein was estimated by kjeldhal method, Carbohydrates were determined by difference method.[18-22] (Tables 1 and 2)



Flow sheet diagram of dehydrated apricot.

S #	Parameter	Result
1	Moisture	83.3
2	Ash	0.72
3	Carbohydrate	14.03
4	Protein	0.9
5	Fat	0.03
6	Fiber	1.02

Table 1: Chemical composition of fresh apricot halman (gm/100 gm).

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S #	Parameter	Results	
		Moveable solar drier dehydrated apricot (%)	Open Sun dehydrated apricot (%)
1	Moisture	14.61	15.7
2	Ash	3.51	3.43
3	Carbohydrates	75.91	75.13
4	Crude Protein	1.0	0.97
5	Crude Fat	1.99	1.82
6	Crude Fiber	2.98	2.95

Table 2: Chemical composition of dehydrated apricot halman (gm/100 gm).

Organoleptic/Sensory Evaluation of Dehydrated Apricot

The organoleptic/sensory evaluation for appearance, color, texture, taste and overall acceptability conducted using nine point hedonic scale in accordance with the method described by Larmond.[23-27] The panel members were selected on the basis of their ability to discriminate and scale abroad range of different attributes of dehydrated apricot. An orientation program was organized for the panel members to brief them the objective of the study. The samples were served to the panelists for organoleptic/sensory analysis. The judges were provided with prescribed questionnaires to record their observation. The information contained on the performa was Larmond nine point hedonic scale i.e. 9=Liked extremely; 8=Liked very much; 7=Liked moderately; 6=Liked slightly; 5=Neither liked nor disliked; 4=Disliked slightly; 3=Disliked moderately; 2=Disliked very much; 1=Disliked extremely. The panelists expectorated the samples and rinsed mouth using distilled water between samples. The experiment was repeated twice and the values are presented as means.[28]

RESULTS AND DISCUSSION

Physicochemical Composition of Dehydrated Apricot Samples

The highest moisture content was recorded in fresh apricot i.e. (83.3%), followed by the open sun drying apricot was found to be (15.7%) whereas, the lowest values (14.61%) was recorded in the moveable solar dehydrated apricot sample and the results are highly significant ($p<0.01$) among the different methods. The highest ash (3.51%) was found in moveable solar dehydrated sample fallowed by open sun drying sample at (3.43%), whereas the lowest (0.72%) ash observed in the fresh apricot sample, which were significantly different from each other. The highest moisture content in fruits makes it ideal for fruit juicing as a supplement. Simultaneously, high moisture content tends to promote microbiological contamination and chemical degradation. The results indicated that the highest mean values (75.91%) carbohydrate was recorded in moveable solar drier dehydrated samples, while in the open sun drying sample ranked 2nd which was observed (75.13%), where as the minimum mean values (14.03%) observed in fresh samples of apricot. The results obtain from dehydrated sample was statistically different as compared to fresh samples. The highest (%) of protein was observed in moveable solar drier dehydrated samples i.e. (1.0%) followed by open sun dehydrated samples (0.97%). The lowest value of protein (%) of apricot (0.9%) was recorded in fresh apricot samples and the results were highly significant. The highest (%) of fat observed in moveable solar drier dehydrated samples i.e. (1.99%) followed by open sun dehydrated samples (1.82%). The lowest value of fat (%) of apricot (0.03%) was recorded in fresh apricot samples and the results were highly significant. The highest (%) of crude fiber was observed in moveable solar drier dehydrated samples i.e. (2.98%) followed by open sun dehydrated samples (2.95%). The lowest value of carbohydrate (%) of apricot (1.02%) was recorded in fresh apricot samples and the results were highly significant.

This study showed that apricot has high moisture (83.3%). It is known that products that have low fat values normally have high moisture contents. Moisture (%) is a widely used parameter in the processing and testing of food. The observed value implies that cauliflower may have a short shelf-life since microorganisms that cause spoilage thrive in foods having high moisture content and also is indicative of low total solids. The high moisture content of apricot is consistent with the report[29] of which a high moisture value for fruits like white mulberry (82.50%) and black mulberry (78.03%) was observed.

The carbohydrate (%) of apricot fresh fruit (14.03%) in this study is low but it is higher than that of the related fruit mulberry (13.83%). Similarly, protein (%) in apricot is (0.9%) is low and similar to these values reported by researchers in other fruits. such as “mulberry” (1.73%). The fat (%) of apricot is (0.03%) which is than that of kale (0.26%).[30] Since fresh and dry apricot fruit has low fat (%), it can be used by individuals as a low caloric diet to reduce weight. The fiber (%) of apricot (1.02%) was found to be lower than some other fruits such as “mulberry” 11.1%. Fiber cleanses the digestive tract, by removing potential carcinogens from the body and prevents the absorption of excess cholesterol.

Fiber also adds bulk to the food and prevents the intake of excess starchy food and may therefore guard against metabolic conditions such as hypercholesterolemia and diabetes mellitus. Fiber can also help to keep blood sugar levels under control.[31,32] Moveable solar dehydrated and open sun dehydrated apricot samples had higher proximate analysis values due to removal of moisture.

Parameters	Open sun dehydrated apricot	Moveable solar drier dehydrated apricot
Appearance	7.5	8.7
color	7.9	8.5
Texture	8.4	8.4
Taste	8.7	8.7
Overall acceptability	7.6	8.7

Table 3: Mean acceptability scores for dehydrated apricot samples.

Organoleptic/Sensory/Evaluation of Dehydrated Apricot Samples

The dehydrated apricots of open sun dehydration and moveable solar drier dehydrated apricot samples were evaluated organoleptically. The samples were graded by numerical scoring, on a nine point hedonic scale. The results of organoleptic evaluation were reported in Table 3. The organoleptic evaluation shows, slightly reduction in the mean score for over all acceptability of open sun dehydrated apricot. Taste and texture of the both dehydration methods remains same. There was a clear difference shown in mean scores of color, appearance and overall acceptability of samples. The open sun dehydrated apricot has a less attractive color, appearance and overall acceptability as compared to portable and moveable solar drier dehydrated apricot that have high scores in color, appearance and overall acceptability. The dehydration completed in open sun in 9 days interval and in moveable solar drier it dehydrated in only 2 days interval. The poor color and appearance of open sun dehydration is due to more time exposing to light and wind as compared to moveable drier. The fewer score in sensory evaluation in overall acceptability of open sun dehydration may be due to dust and color.[29] However the portable and moveable solar drier dehydrated apricot was liked very much by the panel of judges and open sun dehydrated apricot was declared acceptable.

CONCLUSION

The findings of this study show that the moveable solar dehydration and open sun dehydration of apricot fruit are effective in preserving the chemical composition of apricot and preventing deterioration by reducing moisture.[33] In comparison of open sun dehydration and moveable solar dehydration, organoleptic characteristics of open sun dried apricot have a little negative effect on over all acceptability as compared to moveable solar dehydrated apricot. The moveable solar dehydration is so for good on color, taste and over all acceptability. The fruits dehydrated using solar dryer were hygienically more acceptable as compared to open sun dehydration. However; the open sun dried fruits were declared acceptable by the panel of judges for color, taste and overall acceptability.[34]

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Physico-Chemical and Sensory Evaluation of Cooked Fermented Protein Fortified Cassava (*Manihot Esculenta* Crantz) Flour

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ABSTRACT

Background: The aim of this study was to investigate the effects of fermentation with *Lactobacillus plantarum* strain 6710, on protein- and pro-vitamin A-fortified and wild type cassava flours for production of cooked fufu.
Results: Physico-chemical analysis of fufu flour and cooked fufu were accomplished. Pasting properties determined that non-inoculated zeolin fortified cassava flour at 0 h of fermentation will cook rapidly due to its low pasting temperature. Volatiles such as acetic acid, hexanal, nonanal and decanal were detected in most samples. A trained sensory panel determined no impact of sample type on cooked fufu aroma.
Conclusion: This study showed that it is possible to make a product similar to cooked fufu with protein and/or pro-vitamin A fortified cassava flours inoculated with *Lactobacillus plantarum*.

KEYWORDS: Cassava; Fufu; *Lactobacillus plantarum*; Gas chromatography; Sensory.

ABBREVIATIONS: ILTAB: International Laboratory for Tropical Agricultural Biotechnology; MRS: de Man, Rogosa and Sharpe; BPW: Buffered Peptone Water; RVA: Rapid Visco Analyzer; PDMS/DVB: Polydimethylsiloxane/divinylbenzene; IRB: Institutional Review Board; ANOVA: Analysis of variance; AACC: American Association for Clinical Chemistry.

INTRODUCTION

Over 95% of cassava produced in Nigeria is used for human food. However, a cassava root-based diet does not provide complete nutrition because it contains 85% starch and only 1-2% protein. Additionally, toxic compounds and rapid deterioration after harvest limit the utilization of unprocessed cassava.[1]

Fufu is a fermented wet cassava paste that is widely consumed in eastern and south-western Nigeria and other parts of West Africa. However, fufu consumption has decreased due to inherent odor, short shelf life and tedious preparation.[2] Regular fufu is made by fermenting freshly harvested cassava tubers in water in open containers for days and is traditionally sold in the highly perishable wet form.[1] The short shelf life is a serious limitation for large-scale processors. A practical approach for improving the shelf life and marketability of fufu is drying, which is aimed at producing reconstitutable fufu dough with physico-chemical characteristics of cooked wet paste.[2,3] While several methods have been used for the drying of fufu, it was determined that a rotary dryer provided a more acceptable product compared to cabinet and sun drying methods, even though the rotary drying method was not cost effective.[4] In addition, as reported by Sanni et al[2] several drying techniques have been reported to reduce the strong odor of fufu, but the products were sticky, bland and the quality unacceptable when reconstituted from flour.

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The objectives of this study were to determine the characteristics of protein- and pro-vitamin A-fortified fermented cassava flours with or without the addition of *Lactobacillus plantarum* strain 6710 and their influence on the processing of cooked fufu. Similar characteristics for cooked fufu from protein- and pro-vitamin A-fortified cassava and the wild type cassava flour may allow consumers to enrich their diets nutritionally without sacrificing the inherent characteristics of commonly consumed cooked fufu.

MATERIALS AND METHODS

Cassava Flours

Four types of fortified cassava flours (zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO)) with protein contents (wb) of 9.52, 6.83, 3.63 and 2.14%, respectively, as well as wild type cassava flour (1.41% protein), were provided by the International Laboratory for Tropical Agricultural Biotechnology (ILTAB) (St. Louis, MO, USA).

Cultures: Propagation and Storage

L. plantarum BFE 6710 strain grown as a stab culture was provided by the Max Rubner-Institut (Karlsruhe, Germany),[5] and was routinely grown in Lactobacilli de Man, Rogosa and Sharpe (MRS) broth (Difco™, Sparks, MD, USA) at 32 ºC for 24 h underaerobic conditions.

After growth, culture was placed in a cryo tube with a final concentration of 20% glycerol and stored at -72 ºC for further use (stock culture). Working cultures, obtained from stock culture, were streaked on MRS agar, incubated at 32 ºC for 48 h, and colonies transferred to MRS broth. Cultures were propagated twice before use. The pre-culture was centrifuged at 8000 xg at 4 ºC for 10 min. The pellet was washed twice with buffered peptone water (BPW) (BBL™, Sparks, MD, USA), centrifuged and resuspended into 9 mL of BPW resulting in 7×10^{10} CFU/mL.

Growth of Starter Culture in Cassava Flour

All cassava flours were stored in sealed plastic containers at 4 ºC. Ninety grams of flour were transferred to sterilized plastic containers and inoculated with *L. plantarum* by transferring a cell pellet suspension (7×10^{10} CFU/mL). The total moisture content of the cassava sample was adjusted to 68%. A second set of non-inoculated flours was prepared. Non-inoculated (NF) and inoculated flours (LF) were covered and kept at 32 ºC for 96 h.

Fufu Preparation

Fufu flour was prepared as shown in Figure 1. Samples were removed at 0, 24, 48, 72, and 96 h of incubation, and dried for 6.5 h using a tray drier model UOP 8 (Armfield Limited, England, UK) at 70 ºC with air flow of 1.45 m/sec. Dried samples were milled using a Tecator Cemotec 1090 sample mill (Foss-Tecator, Eden Prairie, MN, USA), set to 1, and a portion of the resulting fufu flour was set aside uncooked and stored in plastic scintillation vials at -70 ºC. Milled, fermented cassava samples with and without *Lactobacillus plantarum* strain at all fermentation times were mixed with water in a ratio of 1:3 (cassava: water). Mixes were cooked in a microwave oven Emerson 600 W

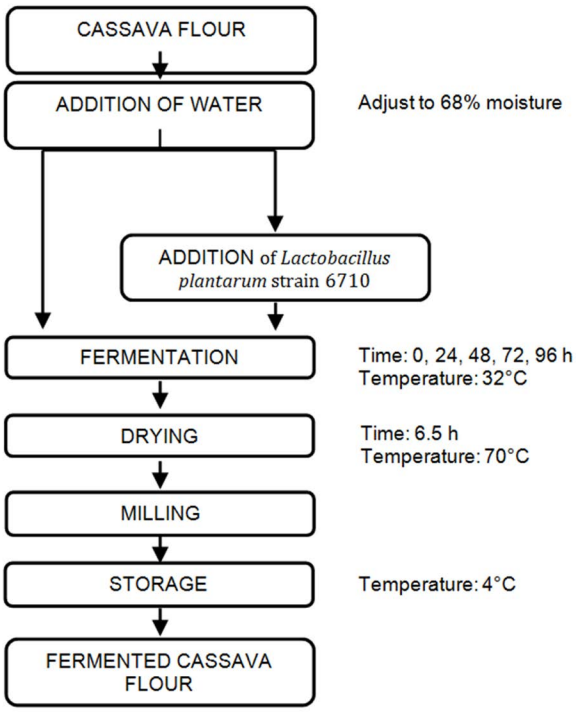

Figure 1: Flow chart for processing of fermented cassava flour.

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(Model MW8665W) at the highest power setting 10(100%) in five cycles of 15 s, each followed by a manual mixing of 1 min. Cooked fufu samples were cooled at 20 ºC for 2 h before sensory evaluation. A portion of the cooked fufu samples was stored at -70 ºC until analysis.

PHYSICO-CHEMICAL ANALYSIS

Pasting Properties

Starch pasting properties were measured for fufu flour with and without added *Lactobacillus plantarum* culture at all fermentation times. The measurements were done with a Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd., Warriewood, Australia) interfaced with a computer equipped with Thermocline software for Windows (release 2.1) according to American Association for Clinical Chemistry (AACC) Approved Method 76-21.01.[6] Heating and cooling cycles were as follows: adjustment to 50 ºC followed by a 1 min hold, heating from 50-95 ºC over 4.42 min, holding at 95 ºC for 2.7 min, cooling to 50 ºC over 3.82 min, and a final hold at 50 ºC for 1.06 min. Peak viscosity, setback viscosity, final viscosity, pasting temperature and time to reach peak viscosity were estimated.[7,8]

Ph and Titratable Acidity (Ta)

Modified methods 943.02, sec. 32.1.20 and 942.15, sec. 37.1.37B were used for determination of pH and titratable acidity, respectively.[9]

Volatile Analysis

The method of Iyer et al[10] with optimization was used for the identification of volatile compounds in cooked fufu samples. Samples (0.5 g) were mixed with sodium chloride (0.33 g) and distilled water (1.0 mL) in 10 mL headspace amber rounded bottom vials sealed with a screw cap possessing a PTFE/silicone septum. The procedure was automated using a CTC CombiPal autosampler (Zwingen, Switzerland), using Cycle Composer software version A.01.04 (Agilent Technologies Inc., CA, USA). Sample was stirred at 250 rpm and a SPME stableflex fiber coated with 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) was exposed to the slurry headspace.

The volatiles were collected for 60 min and thermally desorbed into the injection port of an Agilent Technologies 6890 gas chromatograph with a 6890 N GC split/splitless injector with data collection by Chemstation software version E.02.00.493 and a HP-5MS column (5% Phenyl Methyl Siloxane) (30 m × 0.248 mm × 0.25 µm film thickness) (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas. The injector and detector temperatures were 200 ºC and 250 ºC, respectively. The column temperature was initially at 33 ºC for 5 min before increasing to 50 ºC at a rate of 2 ºC/min, and then to 225 ºC at a rate of 5 ºC/min. The sample was desorbed for 5 min with injector in the splitless mode. Cooked fufu volatiles were identified using a mass spectrometer MS 5975C (inert XL MSD) and MS spectra were compared against a NIST library. Data were quantitated by running standards and developing response factors based on water matrices. The final values were reported as µg/mL.

Color Evaluation

Cooked fufu (~5 g) was placed in a plastic petri dish and the color read on a Minolta colorimeter CM-2002. Values of L*, a*, and b* were recorded.[11] Average values were obtained from two randomly selected points on the surface of the samples.

Instrumental Analysis of Texture

Cooked fufu hardness, adhesiveness and springiness were measured using a TA-XT2 food texture analyzer (Texture Technologies, Surrey, UK) and data were collected with Texture Expert Exceed (Version 2.64). The sample was placed between flat cross heads. A 50 mm diameter flat aluminum cylinder probe along with a flat plate on the HDP/90 heavy duty platform were used for compression of the formed sample spheres. The samples were compressed at cross head speed of 1 mm/s to 50% strain following a trigger force of 0.1 N with a 0.10 s pause between the first and second compressions. Prior to the textural measurements, fufu sample was cooked, set at room temperature for 15 min, weighed (5 g), manually shaped as a sphere and kept at room temperature for 2 hours.

SENSORY ANALYSIS

Sensory Panel Training

Five volunteers (1 male and 4 female), between 18 and 35 years old were recruited from Washington State University based on their availability and consumption of fermented cassava products at least once a week. Panelists were informed that were assessing the sensory properties of fufu. The Washington State University Institutional Review Board (IRB) for human subject participation approved this project.

Panelists were trained for 7.5 h, during which they learned definitions of the attributes, descriptors, and the evaluation technique (Table 1). A 15 cm line scale was used for each descriptor, anchored by the terms ‘low’ (1 cm) and ‘high’ (14 cm). Commercial fufu flour (Kum-Koum) from Cameroon (JKUB, LLC International Foods, and Washington, DC, USA) was used for training.

Formal Sensory Evaluations

Cooked fufu cassava products (5 g) were randomly presented to each panelist, one sample at a time, in a covered 2 oz. soufflé cup identified with a three-digit code. Panelists rated the perception of intensity of selected attributes presented in Table 1 over a period of 7 days. Filtered deionized water was provided for

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ATTRIBUTE/ Descriptors	DEFINITION	TECHNIQUE	STANDARDS
VISUAL			
Color intensity for brownness	The extent to which the color of the sample is intense. The low end of the scale represents a dull brown color and the high end of the scale represents the brown color	Compare to color chips for brownness	Color chips Source: ACE paint colors for your life (ACE N° 9960485). Codes: C20-1 C20-4 C20-2 C20-5 C20-3 C20-6
AROMA			
Fermented aroma	Intensity of fermented cassava aroma	*Swirl the bottle containing the standard. *Remove lid. *2-3 short, sharp sniffs. *Replace lid.	Shredded cassava solution: 1:3 (cassava:water) with 550 µL acetic acid (I:12)
Stale aroma	Intensity of stale aroma	*Swirl the bottle containing the standard. *Remove lid. *2-3 short, sharp sniffs. *Replace lid.	Corked standard solution (Wine awakening): 1 drop of standard + 200 mL water (I:12.5)
Fufu aroma	Intensity of fufu aroma	*Swirl the bottle containing the standard. *Remove lid. *2-3 short, sharp sniffs. *Replace lid.	Fufu flour (Cameroon) solution: 1:3.75 (fufu flour:water) (I:12)
TEXTURE			
Hardness	Force required to fully compress a sample with the fingers	Compress the sample between the thumb and index finger.	Frankfurter (I:13.5)
Adhesiveness	Degree to which a sample adheres to the fingers	Press the sample between the thumb and the fore-and middle fingers, then release; evaluate the degree of adherence.	Cooked fufu flour (Cameroon) solution: 1:3 (flour:water) (I:14)
Springiness	Degree to which sample returns to original shape after a certain time period	Press the sample by the tip of the forefinger and feel the recovery while releasing the finger.	Bread (I:14.5)

Table 1: Attributes, definitions, evaluation technique, standards and intensity values (I) used for sensory evaluation of proposed fufu cassava product.

sniffing between samples. Within each session, 15 flights were presented. A period of 10 min was imposed after the seventh flight to rest the senses. Each cooked fufu product was evaluated in a randomized complete block design. All sessions were carried out in separate booths under white light equipped with a computerized system and sensory software (Compusense® *five* software (5.2, Guelph, ON, Canada) for data collection.

DATA ANALYSIS

Physico-chemical data of dried fermented cassava flour (fufu flour) and/or cooked fufu samples were analyzed for significant differences using a three-way analysis of variance (ANOVA). Tukey's multiple comparisons of means were used to analyze both dried fermented cassava flour (fufu flour) and cooked fufu physico-chemical data with XLSTAT (Version 7.5.3, XLSTAT Addinsoft, France, Paris) at the $p \leq 0.05$ confidence level. The ANOVA performed on physico-chemical data used sample, fermentation, and time as fixed effects. Sensory data of cooked fufu samples were analyzed for significant differences using a three-way analysis of variance (ANOVA) in a randomized complete block using PROC MIXED in SAS 9.1 software (SAS Inst., Cary, NC, USA). The analysis of sensory data using a mixed effects model assumed panelists as a random effect factor and sample, fermentation and time as fixed effect factors. Significance level was defined as $p \leq 0.05$. All tests were duplicated.

RESULTS AND DISCUSSION

pH and Titratable Acidity

Most naturally fermented samples (NF) reached a minimum pH value at 96 h fermentation compared to 72 h for the *L. plantarum* samples (LF). NFS and NF SPRO showed a pH value of 4.34 and 4.51 at 96 and 72 h, respectively. A fast decrease to pH 3.60 was observed when the lactic acid bacteria strain was added for LFS and LF SPRO at 96 and 72 h of fermentation, respectively. These results agree with Brauman et al,[12] who reported that LAB produced a rapid drop in pH to ~4.5 after two days fermentation of cassava roots.

The fermentation process resulted in a gradual increase of acidity for the non-inoculated wild type (NFWT) and inoculated wild type (LFWT) cooked fufu up to 72 h of fermentation, with no further significant change at 96 h ($p > 0.05$). An analogous tendency was shown for the NFZ and LFZ.

When *Lactobacillus plantarum* starter culture was added, the decrease in pH and increase in TA occurred more rapidly (24 h) when compared to samples without starter culture. This agrees with Kostinek et al,[13] who determined a high capability for the obligate homo- and facultative hetero fermentative group (mostly *L. plantarum*) to lower the pH of the medium. Acid pro-

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duction and subsequent decrease extends lag phase of sensitive organisms including foodborne pathogens.[14]

Pasting Properties

Peak time is a property that indicates the minimum time required for fufu cooking. At 0 h fermentation, significant differences in peak time, that may have developed during the pasting analysis of the samples, were found between all NF and LF individual samples of a given cassava material, i.e. NFWT *versus* LFWT ($p \leq 0.05$) (Table 2), with the exception of NF PRO and LF PRO. At 96 h fermentation, NF and LF treatments were significantly different in peak time for all cassava material pairs ($p \leq 0.05$) (Table 2). Peak times were consistently higher for the NF *versus* LF samples (within a given sample pair). This supported the observation that the decrease of pH or increase of acidity occurs more rapidly when *Lactobacillus plantarum* starter culture was added. Therefore, it is likely that fermentation in NF samples was focused most prevalently on amorphous regions of starch granules, which caused an increase of peak time and did not allow a more rapid fermentation. The greatest peak time was found for NF SPRO (4.87 min) at 0 h fermentation, which could be related to protein-carotenoid interaction that increased the time to peak viscosity. Such ingredients may increase the peak time if they coat the granule, limiting water penetration and thus, hydration and swelling. Protein-carotene interaction was shown by Marx et al[15] when crystalline β-carotene with bovine serum albumin in a model system proved to be stable towards isomerization irrespective of time-temperature parameters which indicated binding as the protein protected the carotenoid.

In addition, NF SPRO showed the lowest breakdown values (Table 2). A similar trend was observed for LF SPRO when compared with all inoculated cassava materials at 0 and 96 h fermentation. Therefore, these fufu flours might be able to withstand heating and shear stress compared to the other samples. At 96 h fermentation, NF SPRO also showed the highest peak time (5.14 min) even though it was not significantly different from NFWT, NFS and NF SPRO. Based on the lowest peak time, NFZ and LFZ at 0 and 96 h of fermentation will cook faster with less energy consumed, thereby saving cost and time compared to other samples. Except for NFZ, peak times for NF samples at 96 h fermentation were slightly higher than the values determined for LF samples. This may be due to the conversion of starch to simple sugars due to fermentation by the microorganisms causing a decrease of the stability of the starch materials.[16]

Prior to fermentation, the mean peak viscosity was greatest for NFZ fufu flour (5969 cP) as opposed to NF SPRO (3328 cP). A similar relationship was observed for LFZ and LF SPRO cassava materials (Table 2). This observation might have been influenced by greater or more rapid swelling of starch granules in NFZ, which in turn may cause instability and consequently disruption upon the heating and stirring (i.e., breakdown). Furthermore, the final viscosity and pasting temperature of NFZ flour were substantially lower, likely due to free leaching of amylose and amylopectin from the granules.[17] Thus, it would

Fermentation time (h)[1]	Cassava material[2]		Peak viscosity (cP)	Trough (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Peak time (min)	Pasting temperature (°C)
0	LF	WT	4972±8^{c}	1792±10^{d}	3189±14^{c}	2735±15^{f}	963±54^{c}	3.85±0.03^{f}	64.58±0.67^{d}
		Z	5518±42^{b}	1628±37^{e}	3899±18^{a}	2890±8^{e}	1334±73^{ab}	3.68±0.02^{g}	62.13±0.04^{e}
		S	4285±6^{f}	1672±22^{e}	2620±6^{d}	2588±14^{g}	959±23^{cd}	4.36±0.04^{cd}	71.08±0.11^{a}
		SPRO	3667±32^{g}	1566±13^{e}	2094±8^{g}	2878±16^{e}	1325±21^{ab}	4.46±0.00^{c}	69.78±0.11^{bc}
		PRO	4418±58^{e}	1812±11^{d}	2563±8^{e}	3249±15^{c}	1412±9^{a}	4.26±0.01^{d}	69.05±0.07^{c}
	NF	WT	5476±37^{b}	2790±13^{a}	2652±25^{d}	3949±47^{a}	1194±12^{b}	4.30±0.05^{d}	69.73±0.04^{bc}
		Z	5969±26^{a}	2359±62b	3648±18^{b}	3057±4^{d}	773±40^{d}	4.02±0.04^{e}	61.53±0.04^{e}
		S	4561±40^{d}	1968±28^{c}	2649±11^{d}	2813±17^{ef}	884±44^{c}	4.63±0.04^{b}	65.35±0.07^{d}
		SPRO	3328±2^{h}	1623±8^{e}	1705±6^{h}	2565±25^{g}	955 ±14^{c}	4.87±0.01^{a}	70.35±0.07^{ab}
		PRO	4269±25^{f}	963±25^{c}	2315±14^{f}	3456±11^{b}	1457±35^{a}	4.25±0.02^{d}	69.08±0.11^{c}
96	LF	WT	4965±18^{b}	2243±22^{a}	2740±21^{c}	3244±24^{bc}	1005±6^{c}	4.14±0.01^{d}	60.83±0.04^{b}
		Z	5179±71^{a}	1731±30^{bc}	3453±48^{a}	2707±16^{e}	996±13^{c}	3.77±0.05^{e}	71.03±0.04^{a}
		S	3480±11^{f}	1280±13^{ef}	2189±8^{e}	2240±20^{g}	924±18^{cd}	4.36±0.04^{c}	72.95±0.07^{ab}
		SPRO	2809±81^{g}	1357±59^{e}	1452±21^{f}	2045±13^{h}	775±50^{e}	4.86±0.01^{b}	70.98±0.04^{ab}
		PRO	3633±11^{e}	1045±17^{g}	2590±10^{d}	3240±9^{bc}	2172±24^{a}	4.85±0.02^{b}	70.18±0.25^{ab}
	NF	WT	4571±12^{c}	1779±14^{b}	2818±11^{c}	3303±18^{ab}	1534±18^{b}	5.06±0.01^{a}	62.03±0.04^{b}
		Z	4683±9^{c}	1555±43^{d}	3102±15^{b}	3066±33^{d}	1463±7^{b}	4.03±0.04^{d}	71.60±0.00^{ab}
		S	2830±21^{g}	1633±14^{cd}	1185±18^{g}	3181±7^{c}	1551±11^{b}	5.08±0.02^{a}	75.45±0.07^{ab}
		SPRO	2682±40^{g}	1681±21^{bc}	1051±10^{h}	2489±3^{f}	852±37de	5.14±0.01^{a}	74.23±0.04^{ab}
		PRO	3985±18^{d}	1239±14^{f}	2766±33^{c}	3339±42^{a}	2150±13^{a}	5.05±0.07^{a}	71.03±0.04^{ab}

[1]Results are reported as the mean of two determinations±standard deviation. Means within the same column containing different letters are significantly different ($p \leq 0.05$).
[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.

Table 2: Pasting properties1 of dried fermented cassava flour "fufu flour" with (LF) and without (NF) the addition of a starter culture at 0 and 96 h of fermentation.

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appear that the Z cassava materials swelled earliest and most rapidly, leading to a higher degree of breakdown.

The setback region is the phase of the RVA analysis where starch molecules start to reassociate during cooling, reflecting retrogradation of the starch molecules. Setback has been consequently correlated with the texture of the reconstituted fufu flours.[3,18] Thus, a low setback value indicates low rate of starch retrogradation and syneresis. Here, at 0 h of fermentation the least extent retrogradation was shown with the NFZ fufu flour (Table 2). At 96 h of fermentation, NF PRO and LF PRO fufu flours showed the greatest setback viscosity values possibly due to increased hydrogen bonding during cooling (Table 2). This hydrogen bonding leads to the growth of gel micellar regions, hence increase in index of retrogradation, making entrapped water more prone to expression. This retrogradation as explained by Whistler and BeMiller[18] also depends on other variables that include amylose/amylopectin ratio and their structures that will be based on the botanical source, temperature and starch concentration and other ingredients.

Regarding the final viscosity that indicates the ability of the material to form a viscous paste, NFWT fufu flour showed the highest value at 0 h fermentation while NFWT and NF PRO fufu flours obtained the highest final viscosity values at 96 h (Table 2).

Volatile Compounds

Only a few aromatic compounds were detected in the cooked fufu samples. A similar profile was obtained for all samples in spite of their specific characteristics. The main compounds detected were acetic acid, hexanal, nonanal and decanal at all fermentation times. At 0 h of fermentation, a low concentration of acetic acid was found in all samples (Table 3) and no significant differences were found among treatments (p>0.05). Acetic acid concentrations were greater than the published threshold value (24.3 µg/mL)[19] in all cooked fufu samples fermented from 24-96 h. High concentrations of acetic acid may affect the acceptability of the cooked fufu product. Hexanal was detected in most samples at all fermentation times. No significant differences in hexanal concentration were found among samples at 48 h fermentation (data not shown). In most samples, the level of hexanal was below the published threshold value (0.03 µg/mL).[19] The greatest amount of hexanal was found in NF PRO at 24 h fermentation (0.146 µg/mL) (Table 3).

Due to the cooking process of the fufu flour, small amounts of aromatic compounds were found with exception of the acetic acid, which increased during the process of fermentation. Also, the drying process to obtain fufu flour could have driven off the volatiles which give the characteristic offensive odor of traditionally processed fufu. When microorganisms were used by Fagbemi and Ijah[20] to enrich fufu, the aroma of the developed product was preferred compared to the commercial fufu after 24 h and 48 h of fermentation. However, no determination of aromatic compounds was done. Dougan et al[21] analyzed the aromatic compounds of gari, a fermented cassava based product, before frying. This group found hexanal and nonanal (aldehydes) as we found here in cooked fufu. When Amoa-Awua, Appoh, and Jakobsen[22] determined the aroma profile of the uncooked fermented cassava product agbelima, no influence of the type of inocula used during the fermentation process was found. Then, no information of aromatic compounds has been reported after cooking the product as this is commonly eaten in Africa.

Color Evaluation

Lightness (L*) of NF SPRO and LF SPRO differed significantly (p≤0.05) at 0 h of fermentation with the latter having the greater value. There was mainly a predominant influence of fermentation type rather than sample type. At 48 h of fermentation there were significant differences between NFZ and LFZ; and NF SPRO and LF SPRO (p≤0.05). Significant differences in L*

Cassava material[2]		Volatile compound concentration (µg/mL)[1]							
		0 h				24 h			
		Acetic acid	Hexanal	Nonanal	Decanal	Acetic acid	Hexanal	Nonanal	Decanal
	WT	21.96	nd	0.007	0.003^{b}	91.99^{b}	nd	0.004	0.002
	Z	38.86	0.033^{c}	0.010	0.002^{b}	187.43^{b}	0.006^{d}	0.006	0.002
LF	S	34.05	nd	0.005	0.002^{b}	92.63^{b}	nd	0.005	0.002
	SPRO	41.58	0.045^{c}	0.008	0.002^{b}	133.06^{b}	0.026^{cd}	0.007	0.003
	PRO	20.38	0.086^{a}	0.014	0.002^{b}	196.55^{b}	0.083^{abc}	0.012	0.003
	WT	30.67	0.017^{d}	0.011	nd	117.96^{b}	0.039^{bcd}	0.012	0.002
	Z	32.93	0.013^{d}	0.021	0.006^{a}	298.65^{b}	0.022^{cd}	0.006	0.002
NF	S	35.19	nd	0.004	nd	189.48^{b}	0.013^{d}	0.007	0.003
	SPRO	32.14	0.035^{c}	0.011	0.002^{b}	330.46^{b}	0.095^{ab}	0.010	0.002
	PRO	42.10	0.069^{b}	0.015	0.002^{b}	867.01^{a}	0.146^{a}	0.009	0.002

[1]Results are reported as the mean of two determinations. Means containing different letters within the same column are significantly different (p≤ 0.05).
[2]Wild type (WT), zeolin (Z), sporazein (S), sporazein plus pro-vitamin A (SPRO) and pro-vitamin A (PRO) fortified material.
nd: No detectable

Table 3: Volatile compounds1 detected in cooked fufu with (LF) and without (NF) the addition of a starter culture at 0 h and 24 h of fermentation.

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were also observed for NFWT between 0 and 96 h fermentation with the latter showing less lightness ($p \leq 0.05$). Similar results were visualized for LFWT. NF SPRO presented the greatest mean values for lightness and LF PRO samples at all fermentation times due to the presence of pro-vitamin A that protected the samples from oxidation and loss of lightness. On the other hand, Dziedzoave et al[23] observed an overall decrease of yellow color in agbelima, a cassava fermented product, during the course of fermentation. They stated that may be due to the isomerization of the regular carotenoids present in cassava roots at the onset of acid formation during fermentation. Medoua et al[24] indicated that a decrease of lightness may be due to browning, caused by the oxidation of phenolics compounds, during fermentation. Predominant sugars such as glucose, fructose, and sucrose may also have an influence on the decrease of lightness. Higher protein flours in the presence of sugars may enhance the Maillard reaction or non-enzymatic browning.[25]

Regarding a* values, there was a significant effect of sample and fermentation type. Naturally fermented Z, S and S PRO showed significant differences in a* when compared with their analogous inoculated sample ($p \leq 0.05$) at 0 h fermentation. Similar results were observed at 96 h of fermentation except for PRO sample. Also, the smallest a* mean value was found for NFZ (-2.16) followed by NFWT (-1.91) at 0 h and NFWT (-2.01) followed by NFZ (-1.82) at 96 h. Consequently, a* values of NF and LF WT and Z were negative (green). On the contrary, S and S PRO were positive (red) and PRO samples values changed from negative to positive (green to red) during fermentation.

Samples with pro-vitamin A, SPRO and PRO, became more yellow with time when the starter culture was not added, as recorded by increases in positive b* values. The nutritive value is largely protected during cooking by the carotenoids insolubility in water. However, they are very sensitive to oxidation, which results in both color loss and destruction of vitamin A activity. In this study, fortified pro-vitamin A cooked samples showed an increase of yellow color over time that suggests lack of detrimental effect of the decrease of pH on color. Even though LFS samples did not contain pro-vitamin A, no significant differences were found in b* values between LFS, LF SPRO and PRO samples at 0 h and 96 h fermentation ($p > 0.05$).

Texture Evaluation

In the case of hardness of the cooked fufu samples, no significant differences were observed between samples within each fermentation time ($p > 0.05$). Therefore, the sample type and addition of the starter culture did not play an apparent role in this attribute.

Regarding adhesiveness, no significant effect of addition of the starter culture was observed at 0 h fermentation ($p > 0.05$). This situation changed when significant differences were observed between all samples at all other fermentation times ($p \leq 0.05$). Thus, the composition of the cassava flours significantly influenced the degree of adhesiveness. Even though, similar energy was applied during mixing the cooked fufu samples, it may be possible that mixing intensity could have affected the texture attributes. For example, the breaking up of the gelatinized starch granules in the cooked fufu. The starch leaches out and binds very well with the water added during the pounding process. When the starch granule absorbs water, a good paste is formed and desirable adhesive properties are obtained.

On the other hand, the addition of the starter culture did not influence the adhesiveness of the samples. For example, no significant differences were found on the adhesiveness of the NFWT and LFWT samples at 96 h fermentation ($p > 0.05$). Numfor et al[26] compared the textural properties of starch gels from naturally fermented and inoculated fermented cassava starches. Their results showed that the hardness, gumminess, cohesiveness and elasticity of flour gels were reduced in fermented products. Gel hardness and gumminess have been linked both to the degree of granule swelling and network formation by leached amylose. A reduction in cohesiveness of fermented products has been explained as being due to failure of starch granules to release sufficient amylose. The improvement of textural quality has also been attributed to production of organic acids that complex with soluble amylose.

Lastly, only slight differences in springiness were observed between samples within each fermentation time. In most cases, neither sample type nor fermentation type had a significant effect on springiness. For example, there was a significant difference between the springiness of NFZ and LF PRO samples at 0 h fermentation. At 96 h of fermentation, NFZ was significantly different from NF SPRO, LFWT, LFS, LF SPRO and LF PRO ($p \leq 0.05$).

SENSORY ANALYSIS

Panelists significantly impacted the perception of all cooked fufu attributes ($p \leq 0.05$) (Table 4). There was not a significant effect from sample type, fermentation type, fermentation time and interaction between sample type and fermentation time, fermentation type and fermentation time, interaction between sample type, fermentation type and fermentation time on the fufu descriptor for the aroma attribute ($p > 0.05$). This suggests that using either wild type cassava flour or protein -pro-vitamin A fortified cassava flours, to make a product with a characteristic fufu aroma is feasible.

In addition, no significant effect of fermentation type on fermented aroma was noted. Similarly, no significant effect of fermentation time was found on stale aroma, adhesiveness and springiness texture attributes ($p > 0.05$). However, sample type and fermentation type significantly influenced the perception of texture attributes, hardness, adhesiveness and springiness ($p \leq 0.05$). On the other hand, the interaction between fermentation type and fermentation time did not have a significant effect on hardness, adhesiveness and springiness ($p > 0.05$) (Table 4).

Ray and Sivakumar[27] found no significant differences in texture, color, odor and overall acceptability of fufu ferment-

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Source of Variation	df	Visual	Aroma			Texture		
		Color intensity for brownness	Fermented	Stale	Fufu	Hardness	Adhesiveness	Springiness
Panelist	4	9.59*	294.77***	121.76***	69.39***	234.71***	15.32**	261.31***
Sample Type	4	134.75***	12.65***	4.33**	1.74	15.67***	2.89*	14.03***
Fermentation Type	1	26.60***	0.34	8.72**	0.14	28.25***	7.11**	27.95***
FermentationTime	4	6.40***	6.46***	1.59	1.76	4.96***	1.99	0.26
Sample Type* Fermentation Type	4	5.64**	1.74	0.82	4.45**	3.11*	5.66***	1.16
Sample Type* FermentationTime	16	5.63***	1.21	0.61	0.20	2.65***	1.77*	0.51
FermentationType* FermentationTime	4	6.29***	0.76	0.35	0.43	1.76	1.37	0.28
SampleType*Fermentation Type*FermentationTime	16	3.23***	0.78	0.52	0.67	3.44***	2.40**	0.87

*, **, ***Indicate significant $p \leq 0.05$, 0.01, 0.001 respectively.

Table 4: Degrees of freedom and F-ratios from ANOVA results of trained panel evaluation (n=5) for visual appearance, aroma and texture attributes of cooked fufu with and without *Lactobacillus plantarum* added.

ed from 12 to 96 h. This result disagrees with our findings, where we determined that fermentation time had a significant effect on the brown color intensity, fermented aroma and hardness of cooked fufu ($p \leq 0.05$) (Table 4).

The sensory results indicate that cooked fufu is distinctly different when made from different cassava flour materials in terms of all sensory attributes measured except for the fufu aroma attribute. The characteristic odor of traditionally processed fufu has been attributed to the synthesis of organic acids due to certain hetero-fermentative anaerobic bacteria present in fermenting cassava medium.[28] However here, addition of *L. plantarum* did not affect the characteristic fufu aroma. In an acceptability study, Sobowale et al[29] determined that the addition of a starter culture strain reduced the characteristic aroma in fufu, thereby enhancing a wider acceptability of fufu as compared to the traditional fufu where no culture was added.

According to Sanni et al[2] fufu is considered to be of good quality when it has a smooth texture, characteristic aroma and a creamy-white, grey or yellow color. Based on the color chart used by the panelists, the means color intensity for brownness rating of NFZ and LFZ were lower than the other samples at 0 h of fermentation. This may suggest that Z sample would have a greater acceptability. Thus, Tomlins et al[30] determined that fufu flours should be creamier in appearance to increase their acceptability. At 96 hours of fermentation, the LF SPRO showed the highest mean rating for brown color intensity.

Regarding hardness, no significant differences were found between all NF and LF individual samples of a given cassava material, i.e. NFWT *versus* LFWT ($p>0.05$) except for SPRO. At 96 h of fermentation, NF SPRO cooked fufu showed the highest mean rating for hardness while NFWT had a significantly smaller mean ($p \leq 0.05$). No significant differences were observed for adhesiveness at 72 h and 96 h of fermentation ($p>0.05$). There were significant differences ($p \leq 0.05$) for springiness between NFWT (7.93) and LFWT (10.06) cooked fufu at 72 h of fermentation.

CONCLUSIONS

Lactobacillus plantarum strain 6710 demonstrated its ability to acidify cassava flour during fufu processing. Pasting temperature of the fufu flours established that NFZ (0 h), NFWT/LFWT (96 h) will cook faster than the other samples due to their lower pasting temperature. Fufu flour made from NFZ was more stable as indicated by lowest setback viscosity. Fufu samples generally reached minimum value of pH and maximum acidity at 72 h incubation. Four main aromatic compounds acetic acid, hexanal, nonanal and decanal were detected in most cooked fufu samples at all fermentation times. Color parameters, springiness and adhesiveness texture attributes were most affected by the flour sample type and fermentation time. The sensory results indicated that cooked fufu is distinctly different when made from different cassava flours in terms of all sensory attributes measured. However, the fufu aroma attribute as evaluated by trained sensory panel was not affected by type of cassava flour fermented.

Processing of cooked fufu products with protein and pro-vitamin A fortified cassava flours offer an alternative for fufu consumers; even though a larger acceptability panel would be necessary to indicate the degree of likeness of the developed products.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Research

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Phytoestrogens as Pharma Foods

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ABSTRACT

Phytoestrogens are a diverse group of plant-derived compounds that structurally or functionally mimic mammalian estrogens and show potential benefits for human health. They can serve as potential alternatives to the synthetic selective estrogen receptor modulators which are currently being used in hormone replacement therapy. Estrogens play many important physiological roles in men and women. In women, life is severely affected by a variety of estrogen-related conditions such as osteoporosis, cognitive and cardiovascular disease, increased risk of breast cancer and other symptoms that decrease the overall quality of life. Phytoestrogens are effective in maintaining bone mineral density, prevent bone loss, and help in the prevention and/or treatment of such health related problems. They can be classified as flavonoids, isoflavonoids, coumestans, stilbenes, lignans and terpenoids. The main isoflavones, genistein and daidzein found in soybean, can exist as glucosides or as aglycones, and are readily hydrolyzed in the gut to their aglycones. The aglycones are easily transported across intestinal epithelial cells. Terpenoids (ferutinine, tschimgine, and tschimganidine) found in the Umbelliferae family have estrogenic activities. The main dietary source of phytoestrogenic stilbenes is trans-resveratrol from red wine and peanuts. Plant-derived foods may be an adequate source for a variety of phytoestrogens capable of producing a range of pharmacological effects and protection from various life threatening diseases. This article provides the comprehensive information about the main groups of phytoestrogens, their food as well as herbal or botanical sources, potential health benefits and probable health hazards.

KEYWORDS: Phytoestrogens; Pharma foods; Nutraceuticals; Estrogen antagonists; Flavonoids; Isoflavonoids.

ABBREVIATIONS: DES: Diethylstilbestrol; SECO: Secoisolariciresinol; ER: Estrogen receptors; SHBG: Soy-based infant formulas; PPARs: Peroxisome proliferator-activated receptors; CV: Corn oil vehicle; G: Genistein; SBIFs: Soy-based infant formulas; BD: Beta-defensin-2; S1P: Sphingosine-1-phosphate; CAMP: Cathelicidin antimicrobial peptide; VDR: Vitamin D receptor; SERM: Selective Estrogen Receptor Modulator; CVD: Cardiovascular disease; LDL: Low Density Lipoprotein; HRT: Hormone Replacement Therapy; BMD: Bone Mineral Density; AD: Alzheimer's Disease.

INTRODUCTION

It was observed that Asian populations have lower rates of cardiovascular disease, menopausal symptoms, breast cancer (and other hormone dependent cancers), diabetes and obesity than Western populations.[1] The diet of Asian populations revealed that soy is the major part of food in an Asian diet. This observation has fueled the widely held belief that consumption of soy foods reduces the risk of disease. Phytoestrogens were first observed in 1926,[2] but it was unknown if they could have any effect in human or animal metabolism. In the 1940s, it was noticed for the first time that red clover (a phytoestrogens-rich plant) pastures had effects on the fecundity of grazing sheep.[2,3]

Phytoestrogens as the name suggests are the estrogens (xenoestrogens) that are derived from the plants and not generated within the endocrine system. They can be consumed by

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eating phytoestrogenic plants and so are also known as "dietary estrogens". A phytoestrogen is a plant nutrient that is somewhat similar to the female hormone estrogen. Due to this similarity, lignans may have estrogenic and/or anti-estrogenic effects in the body.

They are a diverse group of naturally occurring non-steroidal plant compounds that because of their structural similarity with estradiol (17-β-estradiol), have the ability to cause estrogenic or/and anti-estrogenic effects,[2] by sitting in and blocking receptor sites against estrogen. Research has shown that phytoestrogens have many health benefits such as reduction in incidence of cardiovascular diseases, prostate cancer and breast cancer. They also provide protection against post menopausal diseases including osteoporosis. Besides, both phytoestrogens such as flavonoids and lignan also possess antioxidant activity.

The major groups of phytoestrogens include flavones, isoflavones, coumestans and lignans. The former three chemically are flavonoids. Phytoestrogens in particular isoflavones are found in high amounts in soybean and their products like tofu whereas lignans are mainly found in flax seed.

Dietary estrogen (phytoestrogen) are found in wide variety of food products (including herbs), even though the level varies depending on the source. The food products with the highest total phytoestrogen content are nuts and oil seeds followed by soy products (Tables 1 and 2). The total phytoestrogen content presented is the sum of isoflavones (genistein, daidzein, glycitein, formononetin), lignans (secoisolariciresinol, matairesinol, pinoresinol, lariciresinol), and coumestan (coumestrol).

Food Sources of Phytoestrogens

The main food sources rich in phytoestrogens are nuts and oilseeds, followed by soy products, cereals and breads, legumes, meat products and other processed foods that may contain soy, vegetables, fruits, alcoholic and nonalcoholic beverages. Flax seed and other oilseeds contained the highest total phytoestrogen content, followed by soybeans and tofu.[4] The highest concentrations of isoflavones are found in soybeans and soybean products followed by legumes, whereas lignans are the primary source of phytoestrogens found in nuts and oilseeds (e.g. flax) and also found in cereals, legumes, fruits and vegetables.

Phytoestrogen (PE) content varies in different foods, and may vary significantly within the same group of foods (e.g. soy beverages, tofu) depending on processing mechanisms and type of soybean used.[5] Legumes (in particular soybeans), whole grain cereals, and some seeds are high in phytoestrogens. Some other examples of foods that contain phytoestrogens are linseed (flax), Sesame seeds, Wheat berries, Fenugreek, Oats, Barley,

Phytoestrogen food sources	Phytoestrogen content (µg/100g)
Flax seed	379380
Soy beans	103920
Tofu	27151
Soy yogurt	10275
Sesame seed	8008
Flax bread	7540
Multigrain bread	4799
Soy milk	2958
Hummus	993
Garlic	604
Mung bean sprouts	495
Dried apricots	445
Alfalfa sprouts	442
Dried dates	329
Sunflower seed	216
Chestnuts	210
Olive oil	181
Almonds	131
Green bean	106
Peanuts	34.5
Onion	32
Blueberry	17.5
Corn	9
Coffee regular	6.3
Water melon	2.9
Milk (cow)	1.2

Table 1: Foods high in phytoestrogen content.

Food items	Total phytoestrogens (µg/100g)
Vegetables	
Soy bean sprouts	790
Garlic	604
Winter squash	115
Green beans	106
Broccoli	94
Cabbage	80
Fruits	
Dried prunes	184
Peaches	65
Strawberry	52
Raspberry	48
Watermelon	2.9
Nuts and other legume seeds	
Pistachios	383
Chestnuts	210
Walnuts	140
Cashews	122
Hazel nuts	108
Lentils	37
Beverages	
Red wine	54
Green tea	13
White wine	12.7
Black tea	8.9
Coffee	5.5
Beer	2.7

Table 2: Total phytoestrogen content in vegetables, fruits, nuts and drinks.

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Beans, Lentils, Yams, Alfalfa, Mung beans, Apples, Carrots, Pomegranates, Wheat germ, Rice bran, Lupin, Kudzu, Coffee, Licorice root, Mint, Ginseng, Hops, Bourbon, Beer, Fennel and Anise, Red clover (sometimes a constituent of green manure).

Due to the molecular similarities with estrogens, phytoestrogens mildly mimic and sometimes act as antagonists of estrogen. Studies have proved that phytoestrogens play an important role in the regulation of cholesterol and the maintenance of proper bone density post-menopause. Evidence is accruing that phytoestrogens may have protective action against diverse health disorders, such as prostate, breast, bowel and other cancers, cardiovascular disease, brain function disorders and osteoporosis.[2,3,6] However, phytoestrogens cannot be considered as nutrients, since the lack of these in the diet does not produce any characteristic deficiency syndrome nor do they participate in any essential biological function.[2]

Phytoestrogens Structure

Chemically phytoestrogens belong to a large group of substituted natural phenolics compounds: the coumestans, prenyl-flavanoids and Isoflavones. These are the three most active estrogenic compounds in this class. Isoflavones are the most researched phytoestrogens and is commonly found in soy and red clover. Apart from this, lignans, stilbenes and terpenoids have also been identified as phytoestrogens but they are not flavonoids.[2] Another term 'mycoestrogens' refers to the mold metabolites of fungus Fusarium that is frequently found in pastures as well as in alfalfa and clover.[7,8] The major phytoestrogens along with their food sources are given in Table 3.

The Major Classes of Phytoestrogens are Discussed below:

Isoflavones

Isoflavones are found exclusively in the family Fabaceae (Leguminosae) and soybeans are a very rich source of them. The isoflavonoids encompass several structurally and biosynthetically related classes such as flavonols, anthocyanins, flavanones, coumestans, and chalcones. Isoflavonoids differ structurally from other classes of flavonoids in having the phenyl ring attached at the 3- rather than at 2-position of the heterocyclic ring. They have similar structure to estrogen and have the capacity to exert both estrogenic and anti-estrogenic effects, they may block the effects of estrogen in some tissues e.g. the breast and womb lining but act like an estrogen in providing possible protection against bone loss and heart diseases. In this subclass, the most thoroughly investigated and interesting compounds with regard to estrogenicity are genistein, daidzein, biochanin A and formononetin. The estrogen effect of isoflavones is much less powerful than the estrogen hormones. This is why isoflavones and phyto-estrogens exercise a balancing effect when the level of estrogens is low, such as during the menopause, and cause less menopause symptoms. A closely related compound to the isoflavonoids is 8-prenyl-naringenin, an isoflavanone, found in hops (*Humulus lupulus*), an ingredient used in beer. Populations in China, Japan, Taiwan and Korea are estimated to consume high quantities of isoflavones and women of these countries complain fewer incidences of osteoporosis and related health problems, especially hot flushes, cardiovascular diseases, lower incidence of hormone dependent breast and uterine cancer.[9]

Flavones

The flavones are a group of naturally occurring chemical compounds widely distributed in the plants. Natural flavones include apigenin, chrysin, quercetogetin, luteolin, and tricetin. Their major food sources are parsley, celery, citrus peels, capsicum, and pepper. Apigenin (4,5,7-trihydroxyflavone) commonly present in fruits and vegetables with proven anti-inflammatory and anticarcinogenic effects in various animal tumor model systems. It has been shown to suppress angiogenesis in melanoma and carcinoma of the breast, skin and colon.[10,11] Apigenin has shown potential to inhibit growth in several human cancer cells, including breast, colon, skin, thyroid, leukemia, and prostate.[9]

Stilbenes

Stilbenes belong to the family of phenylpropanoids and share most of their biosynthesis pathway with chalcones.[12] An example of stilbene is resveratrol found in grapes and has several health benefits. It exists in 2 structural isomeric forms, *cis* and *trans*, with the trans form being more common and possessing greater biological activity. One of the richest sources of this is

Class	Phytoestrogens	Food sources
Isoflavones	Genistein, biochanin A, diadzein (with its metabolites: O-DMA and equol), formononetin, glycetin	Soy, peanut, clover, sunflower seed, walnut
Flavones	Apigenin, chrysin, quercetogetin, luteolin, tricetin	Parsley, celery, citrus peels, capsicum, pepper
Stilbenes	Resveratrol	Grape, peanuts
Lignans	Secoisolariciresinol, matairesional, enterodiol, enterolactone	Soybean, peanut, broccoli, cashew nut, kiwi, pomegranate, triticale straw, flaxseeds, cereals
Coumestans	Coumestrol	Mung beans or soy sprouts, alfalfa sprouts, clover

Table 3: Phytoestrogens of human interest and their food sources.[9]

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Polygonum cuspidatum, a weed that is used in traditional Chinese and Japanese medicines. The primary dietary sources in the human diet are peanuts, grapes and wine. It has exhibited antioxidant, cardio-protective, chemo-preventative, anti-inflammatory, and estrogenic properties, as well as interaction with signal transduction pathways. It has shown to inhibit oxidative-induced apoptosis in a variety of cell lines and reduced oxidative stress in RPE cells. The antioxidant activity of resveratrol may also be associated with protection against the progression of atherosclerosis. The structural similarity of resveratrol to the synthetic estrogen diethylstilbestrol (DES) suggests that it may have estrogenic activity, cardio-protection and prevention of estrogen-dependent cancers. The estrogenic activity of resveratrol may also help prevent bone loss in post-menopausal women.[9,11]

Lignans

The lignan family is a large group of naturally abundant molecules that can be found in a plethora of superior plants where flaxseed is a particularly rich source. Lignans, along with isoflavones and coumestans, comprise the three major classes of phytoestrogens. When plant lignans are consumed, intestinal bacteria convert some into two mammalian lignans, enterolactone and enterodiol. These compounds are absorbed from the digestive tract, circulate and are excreted in the urine.[13-16]

Among lignans, secoisolariciresinol (SECO) and matairesinol are of particular interest. Secoisolariciresinol and matairesinol are two lignan dimers which are not estrogenic by themselves, but readily convert to the mammalian lignans, enterodiol and enterolactone, respectively, which are estrogenic. These are of great interest because of their estrogenic, anti-estrogenic, anti-carcinogenic, antiviral, antifungal and antioxidant activities. Particularly abundant in flaxseed, these molecules can also be found, for example, in soybean, peanut, broccoli, cashew nut, kiwi[17] and pomegranate,[14] triticale straw,[15] greater burdock[18] or *Forsythia intermedia*, asparagus, whole grains and tea.[9] Due to the structural similarity of enterolignans with mammalian oestrogens, these compounds are potentially interesting for combating some hormone-dependent cancers.[19-22] Some epidemiologic investigations have shown that the risk of breast, prostate and colon cancers is lower in countries or regions in which the diet is particularly rich in lignans.

Coumestans

Coumestans are another important group of plant (family Fabaceae) phenols that show estrogenic activity. The main coumestans with phytoestrogenic effects are coumestrol and 4′-methoxycoumestrol. Coumestrol was first isolated from ladino clover (*Trifolium repens* L.), strawberry clover (*Trifolium fragiferum* L.) and alfalfa (*Medicago sativa* L.). Coumestrol and genistein have higher binding affinities to ER-β than the other phytoestrogen compounds. Under *in vitro* conditions, coumestrol has been reported to inhibit bone resorption and to stimulate bone mineralization. Coumestans are less common in the human diet than isoflavones yet similar to isoflavones, in that they are also found in legumes, particularly sprouts of alfalfa and mung bean (*Vigna radiata*) and they are especially high in clover however, low levels have been reported in brussel sprouts and spinach.[9]

Terpenoids

Ikeda et al[23] surveyed estrogenic and antiestrogenic activities of terpenoids phytochemicals found in the Umbelliferae family and revealed that three compounds tschimgine, tschimganidine and ferutinine have agonistic and/or antagonistic activities for ER-α and ER-β. Ferutinine and tschimganidine are sesquiterpenoids and tschimgine is a monoterpenoid. Ferutinine isolated from *Ferula jaeschkeana* was reported to increase uterine weight and prevent pregnancy when administered orally in rats. It may modulate estrogen signaling similar to phytoestrogens specifically, estrogen receptor subtype selective PE and may be useful as natural SERMs.[9,24,25]

MODE OF ACTION

Phytoestrogens bind to the specific receptor sites known as estrogen receptors (ER). These receptor sites are of two types, alpha (ER-α) and beta (ER-β). Generally phytoestrogens display higher affinity for ER-β compared to ER-α.[26] The high affinity of phytoestrogens to estrogen receptors is due to their unique structural configuration that enables them to display estradiol-like effects.[2]

1. Phytoestrogens possess a phenolic ring for binding to estrogen receptor.
2. They have a low molecular weight similar to estrogens (mol. wt. 272).
3. Phytoestrogens possess a ring of isoflavones that mimics the ring of estrogens at the receptors binding site.
4. The distance between two hydroxyl groups at the isoflavones nucleus is similar to that occurring in estradiol.
5. There is an optimal hydroxylation pattern that favours binding with estrogen receptors.

Phytoestrogens also modulate the concentration of endogenous estrogens by binding or inactivating some enzymes and also affect the bioavailability of sex hormones by depressing or stimulating the synthesis of sex hormone binding globulin (SHBG).[3] Research has shown that phytoestrogens bind and transactivate peroxisome proliferator-activated receptors (PPARs). Both ERs and PPARs influence each other and therefore, induce differential effects in a dose-dependent way. The final biological effects of genistein are determined by the balance among these pleiotrophic actions.[27]

Ecology of Phytoestrogens

Phytoestrogens are naturally occurring substances since ancient times and are involved in plant defense systems particularly against fungi. They function as dietary phytochemicals and are considered co-evolutive with mammals.[9] besides phytoestrogens,

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there are some man-made novel exogenous estrogens known as xenoestrogens. They are used as food additives and in ingredients like cosmetics, plastics and insecticides. Xenoestrogens have environmentally similar effect as phytoestrogens as proved in a study of populations.[28]

Phytoestrogens and Birds

Studies have shown that consumption of plants containing unusual content of phytoestrogens under drought conditions decreases the fertility in quail. It has been found that parrot food available in nature possess weak estrogenic activity. Studies are being conducted on screening methods for environmental estrogens present in manufactured supplementary food, with the purpose to enable reproduction of endangered species.[29]

In another, it was found that developmentally inappropriate exposures to estrogenic compounds are known to alter morphology and function of the reproductive tract in various species. Chickens are continually exposed to the relatively potent estrogenic soy isoflavones through the diet. Previous experiments have demonstrated that the primary soy isoflavone genistein induces proliferation of the chick oviduct. However, information is lacking as to specific reproductive tract developmental effects of genistein exposure in chicks. Experiments were done to compare specific oviduct morphological and functional responses to genistein exposure with responses elicited by a classical estrogen, diethylstilbestrol (DES). To avoid the effects of dietary soy isoflavones, the experimental diets were formulated with dried egg white, rather than the usual soybean meal, as a protein source. 100 one day-old female chicks were assigned evenly to 10 treatments: egg white based diet with daily oral gavages of corn oil vehicle (CV); 1 mg DES; 2.0 mg genistein (G2); 20 mg genistein (G20); or 40 mg genistein (G40). At 15 days of age, half the birds from each treatment received a single injection of 2 mg progesterone in a corn oil vehicle to induce ovalbumin synthesis in the oviduct. The classical oviduct responses to estrogen, induction of progesterone receptor and initiation of ovalbumin synthesis, were examined by immune-histochemistry. At 16 days of age, DES treatment increased oviduct weight and percentage of final body weight as compared to all other treatments ($p<0.05$). Immunohistochemistry of formalin fixed oviduct samples revealed that the DES, G20, and G40 treatments significantly increased specific staining for progesterone receptor and ovalbumin in the chick oviduct as compared to CV and G2 treatments. It was concluded that genistein can function as a classical estrogen in the chick oviduct and that dietary exposures to genistein may alter oviduct development.[30]

Effect of Phytoestrogens on Humans

Phytoestrogens are readily absorbed and circulated in plasma and the unabsorbed portion is finally excreted in the urine. The metabolic pathway of phytoestrogens is completely different in humans as compared in grazing animals. This is due to their difference in digestive systems.[9]

Clinical trials on males have shown no observable changes in testicular or ejaculate volume when their diet was supplemented with isoflavone. A meta-analysis of 15 placebo-controlled studies has also shown that the incorporation of soy foods does not alter the bioavailability of testosterone concentrations in men.[31]

Contrary to this, some epidemiological studies have shown the protective effects of phytoestrogens in females against breast cancer. It has also been found that females with history of breast cancer should consume the soya products with caution since soybean can stimulate the growth of estrogen receptor-positive cells *in vitro*. However, the potential for tumour growth is related only with small concentration of genistein and the protective effect was found to be related with larger concentrations of same phytoestrogens.[5] Although not much information is available on the mechanism of action of isoflavones to inhibit tumour growth, but the *in vitro* studies justify the need to evaluate the impact of isoflavones on breast tissue in females.[32] Epidemiologic studies suggest that consumption of soy estrogens is safe for patients with breast cancer and that it may in fact decrease mortality and recurrence rates.[33] It has been reported that phytoestrogens such as genistein may help to prevent photo aging in human skin and promote formation of hyaluronic acid.[34]

Effect of Phytoestrogens on Infants

It has been found that there are no adverse effects of phytoestrogens on infants.[35] Research has shown that there are no adverse effects on human growth, development, or reproduction due to the consumption of soy-based infant formula as compared to conventional cow-milk formula.[36-37] In a clinical studies of infants fed SBIFs [soy-based infant formulas] have resolved questions and raised no clinical concerns with respect to nutritional adequacy, sexual development, neurobehavioral development, immune development, or thyroid disease. SBIFs provide complete nutrition that adequately supports normal infant growth and development. FDA has accepted SBIFs as safe for use as the sole source of nutrition. Although clinical guidelines published by American Academy of Pediatrics stated that isolated soy protein-based formulas may be used to provide nutrition for normal growth and development, but there are few indications for their use in place of cow milk-based formula. These indications are especially for infants with galactosemia and hereditary lactase deficiency (rare) and in situations where a vegetarian diet is preferred.[38]

Ethnopharmacology of Phytoestrogenic Plants

Phytoestrogenic plants are used in the treatment of menstrual, menopausal and including fertility problems. Phytoestrogen containing plants are *Pueraria mirifica* and its close relative, kudzu, Angelica, fennel and anise. In an another study, phytoestrogens rich plant red clover has been shown to be safe, but ineffective in relieving menopausal symptoms, black cohosh is also used for menopausal symptoms, but does not

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contain phytoestrogens. Panax Ginseng contains phytoestrogens and has been used for menopausal symptoms.[39]

Biological Activities of Phytoestrogens

Antimicrobial activity and Phytoestrogens

In a study, synergistic antimicrobial activities of phytoestrogens were observed in crude extracts of two sesame species against some common pathogenic microorganisms. Methanolic and ethanolic extracts exhibited broad spectrum antimicrobial effect against all the tested pathogenic micro-organisms except *Streptococcus pneumoniae* and *Staphylococcus aureus* respectively, while the aqueous extract exhibited inhibitory activity on *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Candida albicans*. The result confirmed the folkloric claims of the antimicrobial effectiveness of locally consumed sesame leaves extracts especially against bacterial and common skin infection in many areas of Nigeria.[40]

In another study, it was found that soybean phytoestrogen enhances the antimicrobial peptides beta-defensin-2 (BD) synthesis in endometrial epithelial cells with lipopolysaccharides and polyinosinic-polycytidylic acid stimulation. This study provided the first evidence and a role of BD in mucosal defense against pathogen in glandular endometrial epithelium. The differential modulation of the expression and secretion of BD by soybean phytoestrogen could be applied for protection of female reproductive tract from pathogen invasions.[41]

In an another study, it was discovered that a signaling lipid, sphingosine-1-phosphate (S1P), generated by sphingosine kinase 1, regulates a major epidermal antimicrobial peptide's cathelicidin antimicrobial peptide (CAMP)] expression *via* an NF-κB→C/EBPα-dependent pathway, independent of vitamin D receptor (VDR) in epithelial cells. Activation of estrogen receptors (ERs) by either estrogens or phytoestrogens also is known to stimulate S1P production, but it is unknown whether ER activation increases CAMP production. The researchers investigated whether a phytoestrogen, genistein, simulates CAMP expression in keratinocytes, a model of epithelial cells, by either a S1P-dependent mechanism(s) or the alternate VDR-regulated pathway. Exogenous genistein, as well as an ER-β ligand, WAY-200070, increased CAMP mRNA and protein expression in cultured human keratinocytes, while ER-β antagonist, ICI182780, attenuated the expected genistein and WAY-200070-induced increase in CAMP mRNA/protein expression. Genistein treatment increased acidic and alkaline ceramidase expression and cellular S1P levels in parallel with increased S1P lyase inhibition, accounting for increased CAMP production. In contrast, *si*RNA against VDR did not alter genistein-mediated up-regulation of CAMP. Taken together, genistein induces CAMP production *via* an ER-β→S1P→NF-κB→C/EBPα- rather than a VDR-dependent mechanism, illuminating a new role for estrogens in the regulation of epithelial innate immunity and pointing to potential additional benefits of dietary genistein in enhancing cutaneous antimicrobial defense.[34]

Phytoestrogens for Cancer Prevention

Phytoestrogens display an array of pharmacologic properties and investigation of their potential as anticancer agents has increased dramatically. Phytoestrogens have been investigated as natural alternatives to hormone replacement therapy and their potential as chemopreventive agents. Scientists have investigated the effects of equol, genistein and coumestrol on cell growth in fully estrogenized MCF-7 cells, simulating the peri-menopausal state and long term estrogen deprived MCF7:5C cells which simulate the postmenopausal state of a woman after years of estrogen deprivation and compared the effects to that of steroidal estrogens: 17 β-estradiol (E2) and equilin present in conjugated equine estrogen. Steroidal and phytoestrogens induce proliferation of MCF-7 cells at physiologic concentrations but inhibit the growth and induce apoptosis of MCF7:5C cells. Although steroidal and phytoestrogens induce estrogen responsive genes, their anti-proliferative and apoptotic effects are mediated through the estrogen receptor. Knockdown of ER-α using siRNA blocks all estrogen induced apoptosis and growth inhibition. Phytoestrogens induce endoplasmic reticulum stress and inflammatory response stress related genes in a comparable manner as the steroidal estrogens. Inhibition of inflammation using dexamethasone blocked both steroidal and phytoestrogen induced apoptosis and growth inhibition as well as their ability to induce apoptotic genes. Together, this suggests that phytoestrogens can potentially be used as chemopreventive agents in older postmenopausal women but caution should be exercised when used in conjunction with steroidal anti-inflammatory agents due to their anti-apoptotic effects.[42]

In yet another study, it was proved that some phytoestrogenic compounds are associated with reduced risk of endometrial cancer. The development of endometrial cancer is largely related to prolonged exposure to estrogens without cyclic exposure to progesterone. Unopposed estrogens increase mitotic activity in endometrial cells, whereas progesterone reduces this activity. The identification of factors that lower endogenous estrogen levels is therefore, important in efforts to prevent this disease.[43]

Estrogens found in plant foods (phytoestrogens), such as isoflavones found in soybeans and lignans found in whole grains, seeds, and dried fruit, have been shown to lower endogenous estrogen levels. They also stimulate the production of sex hormone–binding globulin (SHBG) by the liver. Higher SHBG levels result in more bound and thus less free estradiol, reducing the amount of estrogens available for binding with estrogen receptors. Phytoestrogens also bind competitively to estrogen receptors, thereby blocking binding by estradiol and other estrogens. Because of their weak estrogenic potential (0.1% that of estradiol), phytoestrogens do not elicit a strong estrogenic response and thus have an antiestrogenic effect that inhibits the growth and proliferation of estrogen-dependent

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cancer cells.[43]

Phytoestrogens for Breast Cancer Treatment

Flaxseed is the richest dietary source of lignans, a type of phytoestrogen. They are found in a variety of foods, including soy, flaxseeds, other nuts and seeds, whole grains, and some vegetables and fruit. Most of the research regarding flaxseed and breast cancer focuses on the lignans found in flaxseeds, and their potential for weak estrogenic or anti-estrogenic effects in a woman's body.[44] Lignans, can also change estrogen metabolism. In postmenopausal women, lignans can cause the body to produce less active forms of estrogen. This is believed to potentially reduce breast cancer risk. There are evidences that adding ground flaxseeds into the diet decreases cell growth in breast tissue as well. This would be the type of change that would be expected to decrease breast cancer risk.[8] It is well known that all cells have the ability to go through a process called apoptosis, or programmed cell death. It is believed that through this process, the body can prevent damaged cells from reproducing, and eventually developing into cancer. Researchers have shown that flaxseed sprouts can increase apoptosis. Some cell and animal studies have shown that two specific phytoestrogens found in lignans, named enterolactone and enterodiol, may help suppress breast tumor growth. Animal studies have shown that both flaxseed oil and lignans can reduce breast tumor growth and spread, even for ER- cancer cells. This result suggests that flaxseeds may have anti-cancer benefits that are unrelated to any type of effect on estrogen or estrogen metabolism.[45]

Tamoxifen is a medication known as a Selective Estrogen Receptor Modulator (SERM). It binds with estrogen receptors, without activating growth in breast cancer cells. In this way, tamoxifen prevents a women's own estrogen from binding with these cells. As a result, breast cancer cell growth is blocked.[46] One study in mice concluded that flaxseed inhibited the growth of human estrogen-dependent breast cancer, and strengthened the tumor-inhibitory effect of tamoxifen. Multiple other studies with mice have shown that dietary flaxseed works with tamoxifen to inhibit breast tumor growth.[47] Researchers are not confirmed about the results will apply to women with breast cancer, but this approach of adding flaxseeds to the diet looks promising. And several studies in women have shown that higher intake of lignans, the key phytoestrogen in flaxseeds, is associated with reduced risk of breast cancer.[48] Further, lignans in the diet are associated with less aggressive tumor characteristics in women who have been diagnosed with breast cancer. In other words, women who have already been eating lignans at the time of diagnosis seem to have tumors that are less aggressive.[49]

Phytoestrogens for Preventing Post Menopausal Osteoporosis

Aging causes the progressive loss of bone-mineral density, a process that accelerates during pre-menopause and increases fracture risk. Postmenopausal osteoporosis has become a social problem and it requires appropriate management strategies. Replacement therapy is effective for both prevention and therapy, but recent findings have shown that its long term administration is not as safe as was previously thought, so alternative treatments are urgently needed. Dietary phytoestrogens are emerging as a valid alternative to estrogens in the treatment of menopause-related diseases, such as the climacteric syndrome, cardiovascular diseases, osteoporosis, and dementia. Research has shown that dietary changes in Western habits favoring an increased intake of phytoestrogens-rich foods, could contribute to prevent and to reduce the incidence of postmenopausal osteoporosis in this population.[9]

Phytoestrogens promote estrogenic actions in mammals. They not only act estrogenically as estrogen agonists, but also anti-estrogenically as antagonists by blocking or altering ERs, thus they more closely resemble natural Selective Estrogen Receptor Modulators (SERMS). In short, they perform a complex function as agonists or antagonists depending on the tissue, ER type and quantity and the endogenous hormonal milieu.[5]

In vitro studies have shown that phytoestrogens can be the ideal candidates for treatment of osteoporosis because they are able to stimulate osteoblastic activity and inhibit osteoclast formation. This double positive action is obtained at a range of concentrations (10^{-5} to10^{-7} M) consistent with human ingestion of genistein.[50] The discovery of ERα and ERβ receptors in the bone, the positive effect of selective SERMs such as raloxifene in animals and in humans, and the fact that by virtue of their similarity to raloxifene in forming bonds with the estrogen receptors, phytoestrogens such as genistein, have selective effects on the bone. This protective effect of the phytoestrogens on the bone is produced through the binding of these substances to the estrogen receptors and particularly ER-β ER-β expression is increased during bone mineralization and the high affinity of genistein towards ER-β could make its action efficient at physiological levels.[9] Animal studies have shown that numerous phytoestrogens including coumestrol, genistein, daidzein and others have bone sparing effects in the rat.[51]

Evidence for measurable effects in humans is equally mixed. At least one study has found that post-menopausal women consuming high quantities of soy foods have better femoral and/or lumbar spine density compared to women who consume less soy.[52] A 2009 meta-analysis of randomized clinical trials conducted in humans, however, found only a weak association between increased consumption of soy isoflavones and improved bone-mineral density, leading the authors to conclude that soy isoflavones were unlikely to meaningfully reduce the risk of osteoporosis.[53] Thus adding soya to diet can help stave off bone loss in mid-life especially for women.

Phytoestrogens for Prostate Cancer

Worldwide disparities exist between geographic regions with regard to prostate cancer incidence and mortality. Countries in East Asia have lower rates of prostate cancer compared with Western countries such as Canada and the US. Some suggest

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that dietary differences between the two geographic regions, particularly the higher amount of phytoestrogens consumed in East Asia, is responsible for the difference in prostate cancer incidence. Phytoestrogens are hormonally active compounds present in plant foods that are being studied extensively for their potential roles in hormonally-sensitive neoplasms such as prostate cancer. The mechanism of action of the soy isoflavones is incompletely understood, but in regards to prostate carcinogenesis likely involves estrogenic effects, cell cycle inhibition, anti-angiogenesis and induction of apoptosis. Recent clinical studies have provided mixed results with regard to a clear association between prostate cancer and soy consumption.[54]

The evidence for a protective role of phytoestrogens is not conclusive enough for a general recommendation for their use as dietary supplements, but phytoestrogens can be considered for therapeutic use in prostate cancer patients under certain circumstances. A literature review was performed to study the evidence regarding the chemo-preventive role of phytoestrogens in healthy men, the protective role in early prostate cancer, and a possible therapeutic role in advanced prostate cancer patients. Dietary supplementation with phytoestrogens for chemoprevention of prostate cancer is still a debatable subject. Numerous pre-clinical *in vitro* studies have been promising, and novel molecular mechanisms of action for phytoestrogens continue to be identified. However, human clinical trials including studies done on prostate biomarkers and on the effects of phytoestrogens on steroid hormones are complicated by the possibility of local paracrine effects in prostatic tissue by phytoestrogens that are steroid-like in structure. Their interaction with multiple enzymes represents a paradigm for the complexity of phytoestrogen effects and a window into a potential reason that study results are inconsistent or difficult to explain. A final outcome of the phytoestrogen effect in the intact human may be difficult to discern because these agents can inhibit or induce enzymes, destroy cancer cells, yet will have intrinsic estrogenic effects themselves. Larger multi-center, multi-national, randomized controlled trials are needed before definitive recommendations can be made on the usefulness of phytoestrogens for chemoprevention and therapy for prostate cancer. However, combinations of phytoestrogens with radiation therapy and other antioxidants in advanced or metastatic prostate cancer can be considered because there are limited effective therapy options for this group of patients.[55]

In a study, a large meta-analysis suggests that both fermented and non-fermented soy is protective against cancer.[56] While tofu was the only individual food showing a protective effect, the phytoestrogens genistein and daidzein were also associated with a lower risk of prostate cancer. Further evidence of the protective effect of genistein can be concluded from studies using rodent models and human cell lines.

Mentor-Marcel et al[57] investigated the effects of genistein on the progression of prostate cancer in the TRAMP mouse model. When dietary genistein was used to elevate mouse serum genistein to levels comparable to that of Asian men, the rate of poorly differentiated adenocarcinoma decreased in a dose-dependent manner, while survival improved as a function of decreased tumor burden.[57]

In yet another study, using a rat hormonal carcinogenesis model has shown that a soy isoflavone mixture that includes genistein and diazein is able to protect against carcinogenesis in the dorsolateral and anterior prostate lobes.[58] *In vitro* studies revealed that genistein inhibited growth of two prostate cancer cell lines alone or in combination with selenium.[59] The treatment also induced apoptosis through caspase-dependent pathways and reduced expression of matrix metalloproteinase 2, which has been associated with active invasion and metastases.

Phytoestrogens for Cardiovascular Diseases

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality in men and women worldwide. According to the WHO, by 2015, almost 20 million people will die from CVD each year.[60] In menopause the risk of CVD greatly increases due to the loss of estrogen. Lipid profiles, vascular reactivity, cellular proliferation and thrombosis are factors that affect CVD and on which phytoestogens have shown beneficial effects.[61] Mechanisms suggested explaining the prevention of CVD and the reduction of atherosclerosis are: improvement of plasma lipid concentrations, reduction of thrombus formation such as inhibition of platelet action, improvement of systemic arterial compliance and antioxidant activity. Studies suggest that isoflavones as antioxidants may affect atherogenesis by reducing the oxidation of LDL. Phytoestrogens are a subcategory of compounds called flavonoids, a group composed of hundreds or more types of molecules. The 2 classes of phytoestrogens are isoflavones, notably found in soy products and lignans, present in nuts, fruits, cereal grains, tea, and coffee.[62] Hwang et al[63] reported that extracts of soy, alfalfa and acerola cherry (*Malpighia glabra* L., Malpighiaceae) may synergistically interact to prevent LDL oxidation. Because of their assumed health benefit, isoflavone content is advertised in many foods that contain soybeans, and isoflavones are sold as nutritional supplements.

Phytoestrogens for Relief from Menopausal Symptoms

This is the most widely attributed health benefit of phytoestrogen consumption. Research has shown that intake of phytoestrogens provides relief from menopausal symptoms including hot flushes and night sweats. Studies have shown that there is a slight reduction in hot flushes and night sweats with phytoestrogen-based treatment. Extracts containing high levels of genistein appeared to reduce the number of daily hot flushes but it needs to be investigated further. Overall no indication suggested that other types of phytoestrogens work any better than no treatment. Moreover, no evidence was found of harmful effects on the lining of the womb, stimulation of the vagina or other adverse effects with short-term use.[64]

The association between phytoestrogen intake and breast

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cancer risk in a large prospective study in a Dutch population with a habitually low phytoestrogen intake was investigated and it was concluded that in Western populations, a high intake of isoflavones or mammalian lignans is not significantly related to breast cancer risk. Despite this uncertainty, dietary supplements continue to be popular, particularly among women seeking a "natural" alternative to hormone replacement therapy.[65] In yet another review it was concluded that no evidence shows a benefit of phytoestrogens enriched or-derived products for menopausal vasomotor symptoms with the exception of products containing a minimum of 30 mg per day of genistein which have been evaluated for upto two years in four studies.[66]

Phytoestrogens for Bone Health and Osteoporosis

Estrogen deficiency is a major risk factor for osteoporosis in postmenopausal women. Although hormone replacement therapy (HRT) has been rampantly used to recompense for the bone loss, but the procedure is coupled with severe adverse effects. Hence, there is a boost in the production of newer synthetic products to ward off the effects of menopause-related osteoporosis. As of today, there are several prescription products available for the treatment of postmenopause osteoporosis; most of these are estrogenic agents and combination products. Plant-derived natural products, mostly phytoestrogens (isoflavones, lignans, coumestanes, stilbenes, and flavonoids) are used to prevent menopause-related depletion in bone mineral density (BMD). Although, a number of papers are published on menopause-related general symptoms, sexual dysfunction, cardiovascular diseases, Alzheimer's disease, diabetes, colon and breast cancers, there is paucity of literature on the accompanying osteoporosis and its treatment.[67] In a recent study, the effect of soy protein with and without phytoestrogens on bone turnover was determined in post menopausal women i.e. within two years of the onset of menopause when the bone loss is at its greatest. It was found that there was a significant decrease in bone turn over markers of resorption and formation after supplementation with 15 g soy protein and phytoestrogens for 6 months. An initial effect on osteoclast followed by decreased osteoblast function may have beneficial effects on bone health. There was no significant change in bone turn over makers with 30 g soy protein alone for 6 months.[68]

Phytoestrogens and Cognition

Cognition and memory functioning have been reported to decrease around menopause and therefore, studies have investigated the association of ERT and cognition, as well as phytoestrogens and cognition. However, limited studies are available on the effects of phytoestrogens on cognitive functioning. The mechanisms are not understood, but it has been suggested that phytoestrogens act as estrogen agonists and may increase spine density and synapse formation in the hippocampus of adults. In addition, phytoestrogens may interact with the transcription of neurotrophin genes.[69]

Neuroprotective effects of phytoestrogen compounds found in soy have been demonstrated in animal research and cell culture studies. In particular, phytoestrogens have been shown to reduce Alzheimer's Disease (AD) related pathology, potentially alleviating risk of AD progression. In addition to their antioxidant properties, soy products also have the ability to affect cognition *via* interaction with estrogen receptors. However, observational studies and randomized controlled trials in humans have resulted in inconclusive findings within this domain. There are several possible reasons for these discrepant data. Studies which report no effect of phytoestrogens on cognition have mainly been carried out in European cohorts, with an average low dietary consumption. In contrast, investigation of Asian populations, with a higher general intake of tofu (a non-fermented soy product) has shown negative associations with cognitive function in those over the age of 65. Consideration of type of soy product is important, as in the latter sample, protective effects of tempeh (fermented soy) were also observed. Limited data provide evidence that effects of phytoestrogens on cognition may be modified by dosage, duration of consumption and cognitive test used. Additionally, characteristics of the study population including age, gender, ethnicity and menopausal status appear to be mediating variables. Phytoestrogen treatment interventions have also shown time-limited positive effects on cognition. These findings are consistent with estrogen treatment studies, where initial positive short-term cognitive effects may occur, which reverse with long-term continuous use in elderly women. Well controlled, large scale studies are needed to assess the effects of phytoestrogens on the aging brain and provide further understanding of this association.[70]

Phytoestrogens: Side Effects

As it is already known, phytoestrogens are structurally similar to endogenous estrogens such as 17 β-estradiol and are produced by plants. The most well-understood phytoestrogen action on animal physiology, due to ingestion or exposure to contaminated water, involves competitive binding to estrogen receptors. Because of this ability, some phytoestrogens have documented medicinal potential,[71] but in uncontrolled conditions they may adversely affect reproduction.[72] Furthermore, phytoestrogens may also interfere directly with steroid biosynthesis, intracellular signaling, cell proliferation, and gene expression,[73] which has raised concerns in the medical community about their safety.[8]

Consequences of exposure to these compounds are still unclear, as phytoestrogens have been reported to have estrogenic as well as anti-estrogenic effects on vertebrates.[74] Generally, phytoestrogens are considered safe for humans at common exposure levels, such as those found in soy products, but the large-scale anthropogenic production of phytoestrogens in runoff from agricultural areas, wood pulp mill discharge, and sewage treatment plant effluent may still pose a threat to aquatic ecosystems.[75]

In another study, a meta-analysis of side effects was performed comparing phytoestrogen treatment with placebo or no treatment in randomized controlled trials and it was found

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that phytoestrogen supplements have a safe side-effect profile with moderately elevated rates of gastrointestinal side effects. It was also found in the investigation studies that rates of vaginal bleeding, endometrial hyperplasia, endometrial cancer and breast cancer were not significantly increased among phytoestrogen users.[76]

CONCLUSION

Thus from the foregoing it is quite evident that diets rich in plant-derived products may supply a variety of phytoestrogens capable of producing a range of pharmacological effects in the human body. As people live longer, women are spending more of their lives in menopause, affected by a variety of estrogen-related conditions such as osteoporosis, cognitive and cardiovascular disease, increased risk of breast cancer and other symptoms that decrease the overall quality of life. Epidemiological evidence and experimental data from animal studies are highly suggestive of the beneficial effects of phytoestrogens on human health and their potential to be used as pharma foods, but the clinical data supportive of such effects are either not available, or are awaiting design and execution of appropriate prospective large-scale clinical studies. Due to the functional and structural differences of phytoestrogens, their biological activities are also highly variable and there may be other effects that have not yet been studied. Future research should focus on specific soy components, variability in phytoestrogen metabolism and effects of phytoestrogens on specific target tissues.

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ETHICAL ISSUES

There is none to be declared

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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Research

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Crambe (*Crambe Hispanica* Subsp. *Abyssinica*) Grains Mycobiota and Natural Occurrence of Aflatoxins, Ochratoxin A, Fumonisin B_1 and Zearalenone

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ABSTRACT

Crambe grains are a new feed with high concentrations of proteins and fibers. As there is no control during the pre-harvesting or post-harvesting stages of production other grains, crambe may be contaminated by fungi. Fungal overgrowth may lead to mycotoxins production and nutritional properties decrease of the grains. The aim of this study was to analyze the occurrence of fungi and mycotoxins according to pre-harvesting management. Fungal concentration was higher than that recommended by international regulations (3.4×10^6 to 1.3×10^4 CFU.g^{-1}), suggesting that management in pre-harvesting stages of crambe grains production may expose the animals that will feed on these grains to the risk of contamination by fungal toxins. More studies are required about quality of crambe grains, because may be strongly affected by the exposition to variable environmental conditions. But, considering low mycotoxin incidence and levels founded, the crambe proves to be a safe food to be exploited for animal nutrition.

KEYWORDS: Crambe; Aflatoxins; Ochratoxin A; Fumonisin B_1; Zearalenone; Forage.

ABBREVIATIONS: Afs: Aflatoxins; OTA: Ochratoxin A; ZEA: Zearalenone; UFRRJ: Universidade Federal Rural do Rio de Janeiro; DRBC: Dichloran, Rose-Bengal Chloramphenicol; HPLC: High-Performance Liquid Chromatography; AOAC: Association of Official Analytical Chemists; LSD: Least Significant Difference; ANOVA: Analysis of variance.

INTRODUCTION

Crambe hispanica subsp. *abyssinica* or *Crambe abyssinica* is an oleaginous subspecies belonging to Brassicaceae family plant, original from Mediterranean region. Crambe was introduced in Brazil for the production of biodiesel and to be used as forage. The inclusion of crambe seeds was expected to correct the dry matter content and also increase the crude protein content of the silage (containing 37% of oil, 21% proteins, 13% fibers, magnesium, potassium and

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other minerals),[1] reducing diet costs and potential environmental impacts from the disposal of this material on the environment.[2] But, the presence of glycosylates and the mycotoxins limit the use of crambe in ruminant diets should be limited to 4%.[3] The US guideline was only founded limiting the rational use.[4]

As happens with other cultures, crambe may be contaminated by fungi, which may jeopardize physical and nutritional features of the plant and produce mycotoxins, dangerous for human and animal health. Among mycotoxins, special attention must be paid to aflatoxins (AFs) and ochratoxin A (OTA), produced respectively by fungi of the genus *Aspergillus*, section. Flavi, such as *Aspergillus flavus* and *A. parasiticus* and by fungi of the genus *Penicillum*.[5] According to the Brazilian regulation, maximum concentration of total AFs in grains is 50 ppb.[6]

Monitoring of grains during pre-harvest and post-harvest stages is important to avoid undesirable fungal overgrowth and mycotoxins productions. During post-harvest stage, the inspection of storage conditions is crucial at this stage, as the same important, may be impossible disregards geographic localization of cultures and production systems.[7] Contamination of seeds by mycotoxins producing fungi is mainly associated to the relation between the plant and its endophytic mycobiota and to other biological interactions.[8] The main interaction that may lead to a contamination of crambe can be those with insects, with the soil,[9] farming practices,[10] adverse meteorological conditions during the final stage of grains ripening or grains genotype.[11] Mycotoxins of *Fusarium* species have been found to cause major damage, especially in cereals, and could frequently be associated with pre-harvest cereal contamination. The importance of climatic and meteorological assessment during cultivation to assess contamination risks and create predictive models to control the incidence of toxigenic fungi and mycotoxins[12] and the delay of harvesting of oleaginous species, such as peanut may cause increase of *A. flavus* contamination of pods, production of aflatoxin G_1 (AFG_1), aflatoxins G_2 (AFG_2) and drying environment may cause higher incidence of *A. flavus* in the seeds.[13]

The aim of this research was study the mycobiota of crambe and the natural incidence of aflatoxins (AFs), ochratoxin A (OTA), fumonisin B_1 (FB_1) and zearalenone (ZEA) according to pre-harvest handling of grains.

MATERIALS AND METHODS

Sampling

The experiment was performed at the laboratories of the "Nucleus of mycological and mycotoxicological researches" (*Núcleo de pesquisas micológicas e micotoxicológicas* (NPMM)) of the Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil. Crambe grains samples came from the experimental field (2 hectares) of the UFRRJ Department of fitotechnic of Seropédica City, State of Rio de Janeiro, Brazil. The samples used had been collected during harvests performed in 2012 and 2013. The samples from the 2012 harvest had grown in areas either with or without residuals of sunflower plants. The samples from the 2013 harvest had grown in areas with different potassium fertilization (0, 15, 30, 60 or 90 kg K_2O/he) and were harvested with different moistures: 45%, 18% or 9%. During the two harvests drying kept under field conditions, the grains were in uncontrolled environment for drying.

Determination of Water Activity

Grain samples were submitted to water activity (a_w) measurement using Aqualab® cx2 equipment (Decagon Devices Inc., USA).

Mycobiota Count, Isolation and Identification

Mycobiota analysis was performed by decimal dilution in plates, 0.1 ml of each dilution was inoculated in three different culture media: Dichloran Glycerol (DG18) agar for xerophylic fungi, Dichloran, Rose-Bengal Chloramphenicol (DRBC) agar for total fungi estimation[14] and Nash-Snyder Agar (NSA) for isolation of *Fusarium* species.[15] Plates were incubated at 25 ºC for seven days. Quantification was performed as CFUs per gram of sample (10 to 100 CFU/g). Some strains were selected for species identification. Species identification was realized according to macroscopic and microscopic features of samples and according to taxonomic tables.[14-16] Direct plating of grains was also performed. Ten grains of crambe were disinfected and directly distributed on plates containing DRBC, DG18 or NSA agar media. Plates were incubated at 25 ºC for seven days. All plates were assessed and results were expressed as percentage of infected grains. Fungal samples isolated by this technique were classified according to the specific taxonomic keys, described above.

Mycotoxicologic Assessment

Detection and quantification of total AFs, OTA, FB_1 and ZEA was performed with purification in specific VICAM® immunoaffinity columns. Duplicate analysis were performed according to the preconized procedures of any immunoaffinity column (Aflatest, Ocratest, Fumotest e Zeatest Vicam®, Watertown, MA, USA) for HPLC analysis.[17] After samples extraction, screening was performed in a 4ex series VICAM® fluorimeter and successive high-performance liquid chromatography (HPLC) analysis (LC 2000 JASCO® system equipped with fluorescence detector and C18 SUPELCO® silica column). Mobile phase A: water, mobile phase B: acetonitrile/water (95:5, v/v), the flow rate was 50 µL/min. The sample was reconstituted in 400 µL of the starting mobile phase, filtered and 20 µL were injected. External standards for comparison (OTA, FB_1, ZEA and AFs) were calibrated according to Association of Official Analytical Chemists (AOAC), to establishes the detection and quantification limits.

Statistical Analysis

Analysis of variance (ANOVA) was performed to analyze the data. Before performing ANOVA analysis, all data were transformed in Log 10(x+1). Duncan test was performed to compare

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fungal concentration in the different culture media. Least Significant Difference (LSD) Fisher test was chosen to compare mycotoxins quantification data. All statistical analysis were performed using SAS GLM procedure (PROC GLM) (SAS Institute, Cary, NC, USA).

RESULTS

A high variability and heterogeneity of the parameters along the year and moistures percent from studied samples was observed. The a_w, temperatures and precipitation submitted to grains in the different handlings varied, as described: 0.909-0.592, 23.00 °C - 19.00 °C and 25.00-0.20 mm respectively (Table 1).

The mycological research determinate a fungal incidence, with a count in DRBC, DG18 and NSA media varied from 3.4×10^6 to 1.3×10^4 CFU.g^{-1} (Table 2) and the filamentous fungi were predominant over yeasts with an isolated frequency of 65 fungi strains belonging to genera *Aspergillus*, *Fusarium, Penicillium, Cladosporium, Eurotium, Alternaria* and *Curvularia* spp. (Table 3). Couldn´t observes significant difference ($p\geq0.05$) among plate counts of samples for the compared parameters (fertilization and residuals on the ground).

From these samples, were identified the main mycotoxins producing species: genus *Aspergillus*: *A. flavus*, *A. parasiticus*, *A. oryzae*, *A. fumigatus*, *A. niger* aggregated. From genus *Penicillium* were isolated the species: *P. citrinum*, *P. clavatus* and from genus *Fusarium: F. verticillioides e F. chlmydosporum* (Table 3).

Mycotoxins research presents the detected several mycotoxins in studied samples: AFB_1(39.00-4.23 µg.Kg^{-1}), OTA(4.60-4.01 mg.Kg^{-1}) and ZEA(0.76-0.74 mg.Kg^{-1}) (Table 4).

Samples	a_w	MaxT (°C)	MinT (°C)	Rainfall (mm)
C/R; S/R	0.650	20.3	19.4	0.60 mm
C1	0.909	22.5	21.5	0.80 mm
C2	0.592	23.0	21.3	0.20 mm
C3	0.972	20.0	19.0	25.0 mm

Table 1: Mean data of water activity (a_w), maximum temperature (MaxT), minimum temperature (MinT) and rainfall for three days prior to taking the samples from the area with (C/R) and without (S/R) sunflower residue and obtained in three times crops (C1, C2 and C3).

Samples	DG18	DRBC	NSA
C/R	$1.2x10^{6\,a}$	$2.3x10^{6\,b}$	$1.3x10^{4\,c}$
S/R	$2.5x10^{6\,a}$	$3.4x10^{6\,b}$	$1.3x10^{4\,c}$
C1	$7.2x10^{5\,a}$	$4.1x10^{5\,b}$	$\leq1.0x10^{2\,c}$
C2	$1.0x10^{6\,a}$	$4.2x10^{5\,b}$	$\leq1.0x10^{2\,c}$
C3	$2.0x10^{5\,a}$	$7.5x10^{5\,b}$	$\leq1.0x10^{2\,c}$

Limit of Detection (LOD): $\leq10^2$ UFC/g.

Table 2: Fungal counts (CFU.g^{-1}) in three culture mediums (DG18, DRBC, NSA) taking the crambe samples from the area with (C/R) and without (S/R) sunflower residue and obtained in three times crops (C1, C2 and C3).

Areas with and without sunflower residues		
Fungal genus	**Number of samples**	**Frequency (%)**
Aspergillus spp.	13	37.1
Eurotium spp.	3	8.6
Penicillium spp.	3	8.6
Cladosporium spp.	3	7.6
Mucor spp.	2	5.7
Fusarium spp.	6	17.1
Curvularia spp.	2	5.7
Alternaria spp.	3	8.6
Scopulariopsis spp.	1	2.5
Total	36	100.0
Different crops		
Fusarium spp.	15	42.85
Chlmydosporum spp.	10	28.57
Alternaria spp.	10	28.57
Total	35	100.0

Table 3: Frequency (%) of fungal genera in crambe sample from the area with and without sunflower residues and three seasons of crops.

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	Aflatoxins ($\mu g.kg^{-1}$)	Ochratoxin A ($\mu g.g^{-1}$)	Zearalenone ($\mu g.g^{-1}$)	Fumonisin B_1 ($\mu g.g^{-1}$)
S/R	23.00	4.10	0.71	≤0.30
C/R	39.00	4.60	0.76	≤0.30
1° crop				
0	≤0.30	≤0.50	≤0.20	≤0.30
15	≤0.30	≤0.50	≤0.20	≤0.30
30	≤0.30	≤0.50	≤0.20	≤0.30
60	≤0.30	≤0.50	≤0.20	≤0.30
90	≤0.30	≤0.50	≤0.20	≤0.30
2° crop				
0	≤0.30	≤0.50	≤0.20	≤0.30
15	≤0.30	≤0.50	≤0.20	≤0.30
30	≤0.30	≤0.50	≤0.20	≤0.30
60	≤0.30	≤0.50	≤0.20	≤0.30
90	≤0.30	≤0.50	≤0.20	≤0.30
3° crop				
0	6.03	≤0.50	≤0.20	≤0.30
15	4.23	≤0.50	≤0.20	≤0.30
20	4.42	≤0.50	≤0.20	≤0.30
60	6.72	≤0.50	≤0.20	≤0.30
90	7.54	≤0.50	≤0.20	≤0.30

LOD: AFs (0.3 $ng.g^{-1}$), OTA (0.5 $\mu g.g^{-1}$), ZEA (0.2 $\mu g.g^{-1}$), FB_1(0.3 $\mu g.g^{-1}$).
LOQ: AFs (0.6 $\mu g.g^{-1}$), OTA (0.5 $\mu g.g^{-1}$), ZEA (0.6 $\mu g.g^{-1}$), FB_1(1.0 $\mu g.g^{-1}$).

Table 4: Mycotoxin levels in crambe seed samples from plants grown in areas with different levels of potassium fertilization taken from 5 seasons of crops (0, 15, 30, 60 and 90) and area with (C/R) and without (S/R) sunflower residues.

DISCUSSION

The precipitation along the years rising the humidity of the grains and a_w may foster fungal contamination of grains, jeopardizing its use and processing.[19,20] The evaluation of these parameters is a good reference for a predictive condition and modulations.[21]

Crambe grains obtained from plants grown in areas with residuals of sunflowers on the ground showed higher counts on DRBC medium compared to the values practiced by Good Manufacturing Practices (GMP)[22] and Brazil. All samples showed higher concentration of fungi than prescribed by international recommendations and regulations. Grains from the third harvest showed higher fungal contamination, probably because of the heavier rainfall during the three days before the harvest (25 mm) as compared to the others (Table 1). The precipitation and harvest time modulate higher fungal counts.[23]

Observed higher count of fungi in DG18 medium in samples of crambe grains from areas with sun flower residual culture and from samples obtained from the second harvest (C2). The higher contamination with fungi in samples from the second harvest could be associated to the dry weather during the three days before harvesting (0.2 mm of rainfall) as compared to the other samplings.

The high concentration of fungi may affect the quality of feed, causing economic losses and damages to animal health. The importance of meteorological study during harvest to assess the risk of contamination by mycotoxins producing fungal species.[24]

Aflatoxins are produced by fungi of the *Aspergillus flavus* groups, such as *Aspergillus flavus* and *A. parasiticus*, common in grains and in areas with high air humidity.[25] The most important abiotic factors which influence growth and AFs, OTA, FB_1 and ZEA production by such spoilage fungi include water availability, temperature and when grain is moist.[26]

Calcination does not affect the population of *Aspergillus* spp. on the ground, and does not prevent the contamination of hulls and peanuts grains**.** Also describe, periods of lower humidity of the ground are associated to higher frequency of *Aspergillus flavus* isolation.[27]

Researches relative of different partitioning mycotoxins in fractions of cereals, when they are milled and used for different purposes, including human and animal consumption. The relevance of the mycotoxins founded are corresponding with European dates, as show a relevance of incidence of AFs, OTA, ZEA, DON and FB_1 in grains.[28]

The relevance of weather forecast and climate knowledge during harvest and point out the importance of drawing sampling plans and predictive models to assess the risk of contamination by fungi in cultivated areas. The quality of crambe grains may be strongly affected by the exposition to variable environmental conditions. More studies are required on the quality

of this feed and continuous analysis are necessary to protect the safety of crambe grains and improve its use for Brazilian herd.

CONCLUSION

In relation to the assessed parameters, considering the low toxin levels found and the use of available material for animal feed, the crambe proves to be a safe food to be exploited for animal nutrition. The high counts of fungi and the presence of detectable mycotoxins in the grains suggest critical situations at pre-harvest stage and needs to review and control this practice. The presence of fungi and mycotoxins in crambe grains associated to the exposure to adverse meteorological conditions may cause a risk of contamination to the animals that will feed on these grains.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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