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Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998;

1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001) References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. *Afr. J. Biotechnol.* 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant *Staphylococcus aureus* in community-acquired skin infections. *Emerg. Infect. Dis.* 11: 928-930.

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

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications.* McGraw-Hill Inc., New York, pp. 591-603.

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Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

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*Review*

# The role of biofortification in the reduction of micronutrient food insecurity in developing countries

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**Micronutrient malnutrition is a global public health problem, especially in developing countries. Hunger and starvation which are causative agents of malnutrition are occasioned by poor food supply and low income purchasing power for the expensive animal sources of micronutrients. Access to adequate, safe and nutritious food required for a healthy and active life by all people at all times is limited, resulting in micronutrient food insecurity. The quantity and quality of food available for consumption to people determine their micronutrient security level. Inadequate quantity and quality of food available for consumption are causative agents for macronutrient and micronutrient deficiencies. Bio-fortification is an emerging method to increase the micronutrient values of crops in order to eradicate hidden hunger in developing nations. This paper therefore describes the contribution of biofortification in fighting micronutrient malnutrition in developing countries.**

**Key words:** Micronutrient food insecurity, biofortification, developing nations, Micronutrients.

## INTRODUCTION

Food insecurity and malnutrition in developing nations is an issue of global concern (IELRC, 2010). The global population size is currently 6 billion, and it is rising rapidly. The United Nations estimates that the world's population will grow to reach 8.1 billion by 2030 (InfoResources, 2006). In 2007, the number of hungry people in the world was said to have increased by 75 million because of rising food prices (FAO, 2008a). The world's hungry people are put at 963 million (Ruane, 2010). Meeting global food requirements at that point will necessitate an increase in production by 50%. If natural resources are continually used the way they are today, they will not suffice to fuel this increase (InfoResources, 2006).

Developing nations are having challenges of provision of adequate food for their population. For example, Nigeria is in dire need to feed its teeming population of

140 million that is increasing at an annual rate of at least 2% (Egesi, 2010). Poverty, hunger, starvation and malnutrition are grossly prevalent in developing countries. The rate of urbanization is very high in Nigeria. Many youths are rushing to mega cities in search of white collar jobs yet the jobs in these cities are not enough to go round due to population explosion, for example in Lagos state, many youths have resorted to riding a tricycle called "Okada" to make ends meet due to lack of job. At the end, they resort to riding "Okada" which is becoming a road menace despite the alternative means of transportation they provide. Yet these are the productive men that can engage in farming in the rural areas where we have arable farm lands. In urban Nigeria and most of Sub-Saharan Africa, employment in sectors that pay regular wages, such as manufacturing industries, accounts for less than 10% of total employment (Rondinelli

and Kasarda, 1993). The population of many villages now is made up of aged men and women who cannot farm. If they do at all, they maintain small farms around their compounds. So what is going on in most Nigerian villages is subsistence farming and not commercial farming and that can only feed a household and not a population. Even the few farm produces that are available are transported to the urban areas where they will yield more money for the farmer. This makes the left over farm in the villages expensive, sometimes more than what the prices are in the towns. Even the farm produces are not enough for the teaming populace in the urban areas due to over population. Due to the high population of urban areas most of the available lands for farming are inhabited including swamps and canals that would have been viable for local rice farming and vegetables.

Consequently, there is a growing incidence of hunger and malnutrition both in the rural and urban areas even though the former is worst hit. Both rural and urban poor people suffer from food insecurity and poor nutrition, caused in large measure by poverty and lack of nutritional balance in the diet they can afford (Tonukari and Omotor, 2010). Hunger and starvation are some of the reasons why some people are sick in many developing countries. It becomes imperative to increase farm yields in terms of quantity and quality to be able to ameliorate the pang of hunger and starvation in the country. Biotechnology which aims at increasing crop yield, early maturation of farm produces and enriching crops, livestock and fisheries with macro and micro nutrients is one way of eradicating malnutrition in developing nations.

The Nigerian Senate enacted the Biosafety Bill into law on June 1, 2011, after several years of stakeholders' discussion and debate (Ebegba and Gidado, 2011). The passing of the bill is a major step towards the safe and responsible use of biotechnology crops in the country.

### **CONTRIBUTION OF BIOTECHNOLOGY IN FIGHTING MICRONUTRIENT MALNUTRITION**

Biofortification is the genetic or agronomic breeding of crops to enhance their nutritional composition (Uchendu, 2012). Commercial cultivation of genetically modified (GM) crops has been in existence since 1996. About 22 countries are growing GM crops. These include USA, Argentina, Brazil, Canada, China, Paraguay, India, South Africa, Uruguay, Australia, Mexico, Romania, the Philippines, Spain, Colombia, Iran, Honduras, Portugal, Germany, France, Czech Republic, and Nigeria.

Genetic modification technology can boost yields of crops such as cassava, potato, yam and maize, provide resistance to pests and diseases, improve crops' nutritional content and increase their shelf life (Bimbo, 2011). Currently, the most widespread GM crops in the market

are genetically modified varieties of soy, maize, cotton, and canola. An analogous GM rice variety was planted for the first time in 2005, in Iran (InfoResources, 2006). Maize is the most widely-consumed staple food crop in Zambia, but the regular white variety lacks micronutrients, and nearly 50 percent of Zambian children under five suffer from vitamin A deficiency. The improved maize varieties released by HarvestPlus in Zambia can meet up to 25 percent of daily vitamin A needs of the children (Okello, 2013).

Presently, Nigerian scientists and partners are conducting field trials on genetically modified cowpea and cassava (Flake and David, 2010; Ebegba and Gidado, 2011). These are among the important major staple crops in Nigeria and Sub-Saharan Africa as a whole. Table 1 shows the type of genetically modified crops that are being researched upon in various countries. Successful research and development on these crops will result in plenty quality foods and low level food insecurity in developing and some developed countries.

### **NUTRITIONAL CONTRIBUTION OF GENETICALLY MODIFIED FOODS (GMFS)**

Eventual availability of genetically modified (GM) crops into the market will reduce food prices, create variety and plenty food for people to eat in terms of calorie. This will lead to national, state and household food security thus, preventing macro- and micro-nutrient food insecurity. Biotechnology crops only appeared in the market six years ago (James, 2001). Subsequent dates for release of more bio-fortified crops by Harvest Plus are as shown in Table 2.

Biofortified cassava released in Nigeria by the Nigerian National Varietal Release Committee, the vitamin A cassava varieties are named UMUCASS 36, UMUCASS 37, and UMUCASS 38; and are recognized as IITA genotypes TMS 01/1368, TMS 01/1412, and TMS 01/1371, respectively (Obinna, 2012). They can provide upto 25% of the EAR for women and preschool children (Bouis, 2003). Varieties of biofortified orange-fleshed sweetpotato were introduced in Mozambique and Uganda in 2002 and 2007 (Bouis, 2012). Provitamin A maize varieties that can provide up to 25 percent of the EAR for adult women and preschool children were released in Zambia and Nigeria in 2012. Large-scale delivery will begin in 2013. Varieties that can provide up to 50 percent of the EAR are in testing (Bouis et al. 2013).

1. High iron beans have been released in Rwanda and DRC; varieties that can provide an additional 30 percent of the iron EAR for women and preschool children are being disseminated to 250,000 households.
2. High zinc rice is in varietal release testing in Bangladesh and India. Candidate varieties would provide 25% of the

**Table 1.** GM crops being researched upon in developing countries.

Country	Research area
People's Republic of China	Rice, cotton, maize, wheat, and vegetables
India	Rice, maize, cotton, citrus, coffee, mangrove, vanilla, and cardamom
Indonesia	Rice, cassava, maize, cotton, soybean
Malaysia	Rice, papaya, orchid, chili, rubber, and oil palm
Pakistan	Rice, cotton, and chickpea
Philippines	Rice, maize, coconut, mango, and papaya
Thailand	Rice, shrimp, cassava, dairy cows, fruits, and vegetables
Vietnam	Rice, maize, potato, sweet potato, cassava, soybean, sugarcane, and cotton
Nigeria	Cowpea, maize and cassava
Asia	Rice, tropical maize, wheat, Sorghum, millet, banana, cassava, groundnut, oilseed, potato, Sweet potato, and soybean.

Zhang (2000), Sharman (2000), Dart et al. (2001), Nair and Abu Bakar (2001), Zafar (2001), de la Cruz, (2000), Tanticharoen (2000), Tuong-Van Nguyen (2000).

**Table 2.** Release dates for biofortified crops by HarvestPlus.

Crop	Nutrient	Country	Year
Sweet Potato	Vitamin A	Uganda, Mozambique	2007
Cassava	Vitamin A	DR Congo, Nigeria	2011
Bean	Iron	DR Congo	2012
Pearl millet	Iron	India	2012
Maize	Vitamin A	Zambia, Nigeria	2012
Rice	Zinc	Bangladesh, India	2013
Wheat	Zinc	India, Pakistan	2013

Levitt (2011).

zinc EAR for women and preschool children.

3. High zinc wheat is being testing in multilocation trials in both India and Pakistan and the first release is expected in India in 2013 (Bouis, 2003).

Biotechnology's ability to eliminate malnutrition and hunger through production of crops resistant to pests and diseases, having longer shelf-lives, refined textures and flavours, higher yields per units of land and time, tolerant to adverse weather and soil conditions, and generate employment, cannot be over-emphasized (Tonukari and Omotor, 2010). Cassava and white maize are high in carbohydrates but lacks essential micronutrients such as vitamin A.

GM crops have been used to give increased nutritional values to staples. Many of them have been loaded with vitamins and minerals used in fighting 'hidden hunger'. *Hidden hungers* are as a result of lack of vitamins and mineral needed by the body for physiological functions. Hidden hunger can result in micronutrient deficiencies

like vitamin A deficiency, Iron deficiency, zinc deficiency, etc. Biotechnology can be used to alter conventional crop varieties to enhance their micronutrient and protein contents (Mitchell, 2001).

Biofortification provides a truly feasible means of reaching malnourished populations in relatively remote rural areas, delivering naturally-fortified foods to population groups with limited access to commercially-marketed fortified foods that are more readily available in urban areas (Bouis, 2003).

## BIOFORTIFICATION OF CROPS WITH VITAMINS AND MINERALS

### Vitamin A

Vitamin A is a fat-soluble vitamin playing an important role in vision, bone growth, reproduction, and in the maintenance of healthy skin, hair, and mucous membranes



**Figure 1.** Yellow-colored biofortified cassava reveals the  $\beta$ -carotene content. Howe; Maziya-Dixon and Tanumihardjo (2009).



**Figure 2.** Golden Rice.

(FAO/WHO 2002). Vitamin A deficiency (VAD) is a global public health problem in 118 countries, especially in Africa and South-East Asia (Rostami et al., 2007). Vitamin A deficiency is the most common cause of childhood blindness. It is estimated that 228 million children are affected and 500 000 children become partially or totally blind every year as a result of vitamin A deficiency (WHO/FAO, 2003). The geographical areas most affected by vitamin A deficiency are tropical areas where cassava is a staple crop, for example, Brazil, Africa, and Asia (Shrimpton, 1989). Bio-fortification of staple crops with pro-vitamin A carotenoids is an emerging strategy to address the vitamin A status of the poor (Tanumihardjo, 2008; Tanumihardjo et al., 2008).

Bio-fortification breeds crops that are loaded with vitamins and minerals in their seeds and roots. A new approach also supported by the Gates Foundation, World

Bank and the European Commission is Harvest Plus, a biofortification program of the Consultative Group on International Agricultural Research. Netherland is taking a leading role here. An example is Golden rice (Figure 2), a bioengineered pro-vitamin A enriched rice in India, Philippine and Brazil. Up to 73% of energy intake in Asian countries can be from rice. Rice is a staple food in most West African countries, enjoyed by both children and Adults e. g Nigeria. So enrichment of rice with vitamin A has the potential to increase vitamin A intake of vulnerable groups in developing countries. It was suggested that vitamin A contribution from golden rice will provide 50% of the RDA. Biofortification of rice with iron, zinc and lutein are possible. Many research Institutes are developing Golden rice which will have higher vitamin A and iron contents (Mitchell, 2001). Genes are being inserted into rice to make it produce beta-carotene, which the body converts into vitamin A (FAO, 2010). This Golden rice is capable of reducing vitamin A deficiency, anaemia and zinc deficiency which causes childhood and maternal mortality and morbidity. Golden rice was developed by researchers in Germany and Switzerland in 1990s with financial assistance from Rockefeller Foundation (Mackey, 2002). This technology has been transferred to India, South East Asia, China, Africa, and Latin America.

More than 250 million Africans rely on the starchy root crop cassava (*Manihot esculenta*) as their staple source of calories. A typical cassava-based diet, however, provides less than 30% of the minimum daily requirement for protein and only 10 to 20% of that for iron, zinc, and vitamin A (Sayre, 2011). Carotenoid-rich yellow and orange cassava may be a foodstuff for delivering provitamin A to vitamin A depleted populations (Figure 1). Biofortified cassava could alleviate some aspects of food insecurity in developing countries if widely adopted (Montagnac et al. 2009)). Cassava is a target for biofortification because of its importance as a staple crop. It is a staple food and animal feed in tropical and subtropical Africa, Asia, and Latin America. Approximately 500 million people depend on it as a major carbohydrate (energy) source, in part because it yields more energy per hectare than other major crops (Table 3). Cassava is grown in areas where mineral and vitamin deficiencies are widespread, especially in Africa. While cassava was first introduced into Africa (Congo) by Portuguese traders from Brazil in the 16th century, maize was introduced into Africa in the 1500s. These two crops are staple foods in most African countries. Cassava is the primary food staple consumed in the Democratic Republic of Congo (D.R. Congo) and in the humid forest zones of Nigeria. While recent nutritional data are not available for D.R. Congo, a 1998 national nutrition survey indicated that the prevalence of low serum retinol among children 6 to 36 months of age was a tragic 61% (Harvestplus, 2012). In Nigeria, the prevalence of vitamin A deficiency in pre-school children is 29.5%. In both countries, cassava

**Table 3.** Maximum recorded yield and food energy of important tropical staple crops.<sup>a</sup>

Crop	Annual yield (tons/hectare)	Daily energy production (kJ/hectare)
Fresh cassava root	71	1045
Maize grain <sup>b</sup>	20	836
Fresh sweet potato root	65	752
Rice grain	26	652
Sorghum grain	13	477
Wheat grain	12	460
Banana fruit	39	334

<sup>a</sup>Adapted from EL-Sharkawy (2003); <sup>b</sup>All grains reported as dry. Montagnac, J. A., Davis, C. R. and Tanumihardjo, S. A. (2009).

could be a highly effective delivery channel for provitamin A to populations at risk of vitamin A deficiency. HarvestPlus estimates that 10 years after the release of vitamin A fortified cassava, 20 million people in D.R. Congo, and 5 million in Nigeria, will be consuming provitamin A-rich cassava. Cassava is a major carbohydrate staple in Nigeria. It is used in making different delicacies such as eba/garri, fufu/akpu, abacha, etc. The average provitamin A content of cassava is 0.5 ( $\mu\text{g/g}$ ) and HarvestPlus targeted value after biofortification is 15.5 ( $\mu\text{g/g}$ ). This will provide about 7,750  $\mu\text{g RE/kg}$ . Other African countries that are targeted to benefit from this improved vitamin A content of cassava are Republic of Congo, Central Africa Republic, Gabon, Cameroon, Benin, Togo, Ghana, Côte d'Ivoire, Guinea Conakry, Guinea Bissau, Liberia, Sierra Leone, and Angola.

The importance of biofortification of cassava can be seen in its wide production and consumption across African countries. Currently, about half of the world production of cassava is in Africa. Cassava is cultivated in around 40 African countries, stretching through a wide belt from Madagascar in the Southeast to Senegal and to Cape Verde in the Northwest. Around 70 percent of Africa's cassava output is harvested in Nigeria, the Congo and Tanzania (IFAD and FAO, 2000). Throughout the forest and transition zones of Africa, cassava is either a primary staple or a secondary food staple.

Cassava is a primary food staple in the Republic of Congo and secondary food staple in Côte d'Ivoire and Uganda (Nweke, 2012). Maize is the most important cereal crop in Sub-Saharan Africa (SSA) and an important staple food for more than 1.2 billion people in SSA and Latin America (IITA, 2009). Worldwide consumption of maize is more than 116 million tons, with Africa consuming 30% and SSA 21%. However, Lesotho has the largest consumption per capita with 174 kg per year. Eastern and Southern Africa uses 85% of its production as food, while Africa as a whole uses 95%, compared to other world regions that use most of its maize as animal feed. Ninety percent of white maize consumption is in Africa and Central America (IITA, 2009). A marginal nutrient status increases the risk of

morbidity and mortality. Therefore, improving the nutritional value of cassava could alleviate some aspects of hidden hunger, that is, subclinical nutrient deficiencies without overt clinical signs of malnutrition (Montagnac et al., 2009). The Bill and Melinda Gates Foundation have supported a global effort to develop cassava germplasm enriched with bioavailable nutrients since 2005.

The BioCassava Plus initiative has 6 major objectives namely to increase the minerals zinc and iron, vitamins A and E, protein contents and decrease cyanogen content, delay postharvest deterioration, and develop virus-resistant varieties. Using hybridisation and selective breeding, researchers in Nigeria have developed three new yellow varieties of cassava (Figure 3) that naturally produce a higher level of beta-carotene, which they say will help fight malnutrition caused by vitamin A deficiency in the region (Busani, 2011). Orange-fleshed sweet potato has been genetically enhanced to be virus resistant and is being promoted to combat vitamin A deficiency in Kenya, Burkina Faso, Uganda, and South Africa (Vebamba, 2004; Kapinga, et al. 2004; van-Stuijvenberg, 2005). This project has been on in Kenya since 2001 (Mackey, 2002). Papaya (Pawpaw) which was almost decimated in Hawaii was genetically enhanced to resist the ring-spot virus. This virus-resistant technology has also been transferred to papayas in South East Asia, such as Indonesia, Malaysia, the Philippines, Thailand and Vietnam (Mackey, 2002). Papaya is rich in beta-carotene and its consumption can help to eradicate vitamin A deficiency.

Maize is a preferred staple in Africa where the average person consumes over 100 grams a day. Vitamin A deficiency affects over 32% of the African population. Thus, increasing the pro-vitamin A content of maize cultivars may greatly improve the nutrition of millions of Africans (HarvestPlus, 2011).

Private sector collaboration developed the technology to insert the enzymes of phytoene synthase pathway into *Brassica napus* (Canola). Concentrations of 1000 to 1500  $\mu\text{g Carotenoids/g}$  fresh weight of seeds were achieved (Chewmaker et al., 1999). The same technology has been transferred to different species of Canola known as



**Figure 3.** Biofortified cassava. Bill and Melinda Gate Foundation.

*Brassica juncea* (Mustard) (Mackey, 2002). Mustard is widely grown in India, Nepal, and Bangladesh. The oil from the mustard seed is expected to be an excellent source of beta-carotene which can be used in fighting vitamin A deficiency in a vulnerable population.

### Zinc

Zinc content of cereals or food grains have been increased in India by either developing crop cultivars with high concentration of zinc in grains or by adequate zinc fertilization of crops grown on zinc-deficient soils (Rajendra, 2010). Zinc deficiency in preschool children and pregnant women is a public health problem. It can lead to stunted and retarded mental growth. FAO estimated that over 68% of Africa's population is affected by zinc deficiency. Zinc is a secondary nutrient being added into maize by HarvestPlus scientists with considerable success (HarvestPlus, 2011). More than 450 000 infant deaths were recorded in 2004 as a result of zinc deficiency (Rajendra, 2010; Black et al., 2008). Zinc deficiency and Vitamin A deficiency (VAD) coexist in malnourished children (Rahman, 2002). Zinc deficiency limits the bioavailability of vitamin A (Uchendu and Atinmo, 2011).

Rice has demonstrated its ability to be loaded with micronutrients such as vitamin A, zinc, and iron through the work of International agencies, such as Harvest Plus, Humanitarian Board and the International Rice Research Institute in Philippines (Guerta-Quijano et al., 2002). Bioavailability of iron in rice has been increased by inserting a gene for heat resistant phytase from fungal sources that degrades phytate in plants (Bhat and Vasanthi, 2005). This might enhance zinc bioavailability in rice. Impact assessment of this rice will show the

extent of the contribution to zinc RDA of the target population. Golden rice is bio-fortified with pro-vitamin A (beta-carotene) and zinc and is due to be rolled out in the Philippines in 2013 (Levitt, 2011).

### EFFICACY TRIALS WITH BIOFORTIFIED FOOD

Many studies evaluating the efficacy of bio-fortified crops are on-going while some have been completed in countries like Mexico, Nigeria and Rwanda. High iron bio-fortified rice fed to a control group over a period of 9 months was shown to marginally improve the iron status of non-anaemic women in the Philippines (Egli, 2011). Beans have higher iron content than rice and this can be doubled through traditional plant breeding (Beebe et al., 2000).

The major drawback of beans is the low iron bioavailability due to the relatively high content of phytic acid and polyphenols inhibitors (Egli, 2011). About 2% iron absorption has been reported from single meal isotope studies (Donangelo et al., 2003; Beiseigel et al., 2007). Other studies have also confirmed that both phytic acid and polyphenols contribute to the reduced absorption of iron in bio-fortified beans (Petry et al., 2010).

To achieve high amounts of iron absorption from bio-fortified beans, breeding should also focus on reducing phytic acid and polyphenol content. The ability of high  $\beta$ -carotene cassava to prevent vitamin A deficiency has been determined in vitamin A depleted Mongolian gerbils (*Meriones unguiculatus*). Biofortified cassava adequately maintained vitamin A status and was as efficacious as  $\beta$ -carotene supplementation in the gerbil model (Howe et al., 2009).

Biofortified pearl millet bred to contain more iron has been found to provide the recommended dietary requirement of iron for young children. In the study, iron-deficient Indian pre-school children under three years who were fed traditionally-prepared porridges (sheera, uppama) and flat bread (roti) made from iron-rich pearl millet flour absorbed substantially more iron than from ordinary pearl millet flour, enough to meet their physiological requirements. The iron-rich pearl millet also contained more zinc, which was similarly absorbed in sufficient amounts to meet the children's full daily zinc requirements.

Lack of zinc in children can lead to stunting and impaired immune response against common infections (Kodkany et al. 2013). In another study, marginally iron-deficient Beninese women who ate a traditionally prepared iron-rich pearl millet paste were found to absorb twice the amount of iron than paste made from ordinary pearl millet with lower iron content.

The results indicated that less than 160g of iron-rich pearl millet flour daily is enough to provide Beninese women aged 18-45 with more than 70 percent of their daily iron needs. The equivalent amount of the ordinary pearl millet used in the study provided only 20 percent of their iron needs. Women, generally, have higher iron needs

than children (Colin, 2013).

## CHALLENGES FACING GENETICALLY MODIFIED FOODS

A recent forecast estimates that biofortification is more cost-effective than supplementation or fortification in reducing the burden of micronutrient malnutrition, especially in Asia (Meenakshi et al., 2010). Despite this assertion, genetically modified foods are facing challenges of rejection by many poor developing countries. Many of them have doubts regarding the benefits and the safety of biotechnology. In many poor countries the knowhow with regard to biotechnology is very limited, and discussions on risks and advantages are virtually non-existent (InfoResources, 2006). There is also the fear that impacts on health and the environment are not sufficiently demonstrated. For example, Friends of the Earth Nigeria (FoEN), are concerned that bio-fortified cassava undermines biodiversity (Bafana, 2011). Others are worried about the risk of uncontrolled crossbreeding with traditional varieties. They could also be a possibility of toxicity due to overconsumption of these crops and fortified and natural sources of the nutrients. However, this fear is allayed because consumption of beta-carotene unlike vitamin A does not give rise to toxicity because it dose-dependent.

For GM foods to be accepted worldwide, these issues must be addressed. Research results must be disseminated through publications, nutrition education and communication, etc. The positive influence of GM crops on the safety and health of humans, animals, and natural environment must be proved. In the past, GM crops were mainly cultivated and used to produce animal fodders and textiles while small proportion was processed into food. Now that GM foods are emerging as one of the global sources of fighting hunger, starvation and malnutrition, their nutritional quality/value must march that of their natural varieties and even surpass it. The nutritional safety of the products must be guaranteed.

To achieve these, there must be collaboration between the stakeholders to subject biotech produces to continuous and extensive laboratory analyses, evaluation and impact studies to investigate whether the products caused demonstrable effects on the consumers better than the traditional varieties. This will remove doubts regarding the benefits and the safety of biotechnology foods. However, the G8 countries have pledged to improve nutritional outcomes in about 50 million poor Africans and reduce child stunting by "support[ing] the accelerated release, adoption, and consumption of biofortified crop varieties, crop diversification, and related technologies to improve nutritional quality of food in Africa (HarvestPlus, 2012).

Comprehensive research programmes on genetically modified foods are now going on in Argentina, Brazil, China, India and South Africa. Other developing coun-

tries that are implementing biotechnological research programmes on GM crops with a view to commercializing them include Egypt, Indonesia, and Costa Rica. Other countries should follow suit.

## CONCLUSION

Biotechnology is an emerging way to fight malnutrition. In order to realize this objective, genetically modified foods must be affordable for it to substitute the expensive animal products in vulnerable groups. The original physical properties of the traditional crops must not be affected such as taste, flavour, texture, etc.

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Full Length Research Paper

## Genetic differentiation and inheritance of random amplified polymorphic DNA (RAPD) markers in pectoral spine phenotypic sub-groups of *Clarias gariepinus*

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Information on genetic relationship of phenotypically divergent sub-groups would be useful for better identification, utilization and management of species. Recent study revealed phenotypic divergence in a reservoir population of *Clarias gariepinus*. Genetic variability of polymerase chain reaction (PCR) products of the phenotypic divergent sub-groups was investigated in this study. Polymorphism and genetic variability were investigated in electrophoresed random amplified polymorphic DNA (RAPD)-PCR products of blood samples of twenty (20) *C. gariepinus* individuals. The population comprised of five (5) individuals of the non-peses phenotypic sub-group- individuals that did not possess anteriorly serrated pectoral spines denoted by S and fifteen (15) individuals of peses sub-groups that possessed anteriorly serrated pectoral spines denoted as C. Standard protocols were followed in analyzing six screened RAPD primers per individuals DNA fragment. Produced bands of pheno-grams were scored and analyzed to establish polymorphism as well as within and between sub-populations allelic variability using unweighted paired group method of algorithms (UPGMA) and dendrograms cluster analysis. Genotype data of individuals in the groups were tested for canonically significant discriminant grouping using discriminant function analysis (DFA). Results reveal that the primers were polymorphic: 746 bands were obtained from 63 detected loci which gave 80.95% polymorphism. Polymorphic information content (PIC) ranged between 0.18 and 0.49. Percentage polymorphic band were 78.00 and 69.84% for peses and non peses sub-groups, respectively. Dendrogram separated the population to two groups. All peses individuals were in one cluster while all the non-peses individuals were on the second cluster. Within group variations were also observed: DFA revealed that 100% of original phenotypically grouped cases were correctly classified. It was concluded that RAPD primers are suitable genetic markers for establishing variability in *C. gariepinus* sub-populations; the pectoral spine phenotypic groups are genetic variants and are potential varieties for the species. The results would have wide application in identification, utilization and management of genetic resources of *C. gariepinus*.

**Key words:** Random amplified polymorphic DNA (RAPD) marker, morphologic and genetic variability, *Clarias gariepinus*.

### INTRODUCTION

*Clarias gariepinus*, introduced in several countries of Europe and Asia (Saad et al., 2009), has drawn the

attention of aquaculturists because of its biological activities that include faster growth rate, resistance to

diseases and possibility of high stocking density. Species identification and its genetic structure is a crucial issue for the economically important species *C. gariepinus*. Phenotypic studies on specimens of this species in Asejire dam (South-Western Nigeria) revealed presence of two phenotypes that are identifiable by the presence and absence of anteriorly serrated (toothed) pectoral spines. Lather et al. (2010) reported that presence of genetic diversity as well as morphological characteristics in strains proposes a methodology for easy and quick isolation method for both research and industrial analysis. Hence, sustainable utilization of the phenotypic sub-groups would require information on genetic variability of the sub-populations.

Traditional methods like morphological, physiological and biochemical studies used for taxonomic identification are laborious and time consuming (Coulo et al., 1994). Application of molecular markers based on relative difference in deoxyribonucleic acid (DNA) sequences between individuals would detect more polymorphism than morphological and protein-based markers. However, genetic link between the subgroups has not been documented; therefore, we need to use a genetic approach to establish the genetic relationship between the morphologically dissimilar groups. Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction (RAPD-DNA) markers technique generates large number of loci, is less expensive and it requires no prior DNA sequence information to perform the assay (Christopher et al., 2004).

Application of RAPD techniques has greatly increased the ability to understand the genetic relationships within species at the molecular level (Sabir et al., 2012). It was used in establishing clear image about phylogeny and genetic relations between local adaptive breeds in an attempt to generate information for future genetic improvement (El-Rabey and Al-Malki, 2011) and it is a tool for generating taxon-specific markers with different specificities (Day et al., 1997). This technique has been used to assess the genetic variability in animals such as buffalo, cattle, goat, and sheep (Appa Rao et al., 1996), fish (Bardakci and Skibinski, 1994), bacteria (EL Hanafy et al., 2007) and date palm (Soliman et al., 2003). This study utilized RAPD markers in assessing genetic variability of the phenotypically divergent *C. gariepinus* sub-populations.

## MATERIALS AND METHODS

### Sample collection, DNA extraction and RAPD amplification

Twenty (20) live samples of *C. gariepinus* were used for this study. The samples were randomly selected from a collection of the species obtained from Asejire dam (South Western, Nigeria) between

December, 2009–November, 2011. The samples were obtained from catches of a bi-monthly sampling of set gura trap combined with fishermen's landings at the study site. *C. gariepinus* was identified using dorsal and anal fin ray counts following identification key (Teugels, 1986). The samples were re-grouped to two (2) classes based on their phenotypic characteristics. Individuals possessing the anterior serration on their pectoral spines were referred to as peses and were denoted by C while those without the anterior serration were referred to as smooth/non-peses and were denoted by S. Number of individuals selected per group was determined based on their relative proportion in the obtained population. Information on the RAPD DNA analysis is presented in Table 1. Fish identification, phenotypic characterization/sub-grouping and blood collection were carried out at the Department of Aquaculture and Fisheries Management, University of Ibadan, Ibadan, Nigeria. About 2 ml of blood was drawn from vertebral column of individual fish with the aid of hypodermal needle. The drawn blood were released into heparinized sample bottles and transported inside iced container to the Federal University of Agriculture Abeokuta, Nigeria (FUNAAB) where DNA extraction was carried out.

### DNA extraction and dilution

Blood genomic DNA was extracted from the studied individuals using Norgens Blood Genomic DNA Isolation Kit (NORGEN, Biotec Corporation). DNA extraction and purification followed the protocols of Hillis et al. (1996). Quality of DNA was checked by spectrophotometry taking ratio of optical density value at 260–280 nm. 1:100 DNA dilutions was obtained for 10 µl of each extracted DNA.

### PCR mix preparation and gel run

RAPD-PCR product was prepared at the International Institute for Tropical Agriculture (I.I.T.A), Ibadan, Nigeria. PCR-mix contained: 10X Buffer (2.0 µl), 25 mM MgCl<sub>2</sub> (1.6 µl), 5% Tween 20 (2.0 µl), 2.5 mM dNTPs (1.0 µl), 2.0 mM primer (1.0 µl), 5 µl Taq (0.2 µl), water (8.2 µl), diluted DNA (4.0 µl). The PCR mix for each sample was spin down at 10,000 rpm for 30 s inside eppendorf 5415C. Amplification of PCR mix involved denaturation, annealing and extension processes. Thermal cycler (Techne, TC412) was utilized for amplification. The thermal cycle profile comprised of 1 cycle of 3 min initial denaturation at 94°C, 20 s at 94°C, 45 cycles of 20 s at 94°C, 20 s annealing at 37°C, 40 s at 72°C, and 1 cycle of 7 min final extension at 72°C. PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide done under standard electrophoresis procedure. Six (6) random amplified polymorphic DNA primers (OPERON primers) used for this study was obtained from Operon Technologies Inc. Alameda, California, EUA. The primers identities are presented in Table 2. Gel products were photographed and subsequently analysed for polymorphism.

### Determination of polymorphic primers

A set of 20 decamer RAPD primers were initially screened before selecting some of them for this study. Primers screening was carried using three randomly selected samples as template. Presence of polymorphism and clarity of resolution was used in

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**Table 1.** Information on the RAPD-DNA analyzed individuals.

Sample number	Score	Group
1	1	C
2	1	C
3	1	C
4	1	C
5	1	C
6	1	C
7	1	C
8	1	C
9	1	C
10	1	C
11	1	C
12	1	C
13	0	S
14	1	C
15	0	S
16	1	C
17	0	S
18	0	S
19	0	S
20	1	C

Group C individuals had complete anteriorly serrated pectoral spine and their score =1 while group S individuals had smooth anteriorly pectoral spine and their score = 0.

selecting the best 6 primers which were subsequently used for RAPD analysis of the 20 individuals.

#### Band scoring and data analysis

RAPD gel profile of each primer was scored across electrophoretic lanes as variables. Data were recorded as present (1) and absent (0) of band products from the gel electro-phenographs. Polymorphic data analysis followed Lathar et al. (2010). The generated binary data were used to estimate polymorphism level by dividing the polymorphic bands by the total number of scored bands. Polymorphic information content (PIC) was calculated by the formula:  $PIC = 2 \sum P_i (1 - P_i)$ , where  $P_i$  is the frequency of occurrence of polymorphic bands in different primers. Amplified alleles were labeled alphabetically and frequencies of alleles determined. Presence of private allele in the groups and individuals were observed. Establishing genetic differences from the generated data and dendrogram drawn followed the methods of Saad et al. (2009).

Degree of genetic similarity, interrelationship among the studied individuals and calculation of similarity values were carried out using SPSS 15.0- Windows Evaluation Computer Package. The data were analyzed according to binary values 0 and 1. Where, 0=band absence; 1=band present. Results showed both hierarchical pair wise distance using unweighted paired group method of algorithms (UPGMA) and constructed dendrogram. Similarity was observed between all primers and between individuals genotypes with dendrogram constructed in both cases. Genotype data of individuals in the groups were tested for canonically significant discriminant grouping using discriminant function analysis (SPSS,

version 15.0 computer software).

## RESULTS AND DISCUSSION

The population analyzed in the study comprised 5 individuals of the non-peses group (13, 15, 17, 18 and 19) while the rest were in peses sub-group. Table 2 which showed the Polymorphic RAPD primers in *C. gariepinus*: their code, sequence and size range also revealed that the primers were within 150 and 3500 base pairs. Characteristics of the selected primers with respect to the studied population are presented in Plates 1 to 6 while result of the phenograms analysis for polymorphism is shown in Table 3. The result shows that RAPD primers were polymorphic and were able to detect private allele in the studied population. A total of 746 individual bands were obtained from a total of 63 detected loci which gives 80.95% polymorphism. However, the highest number of amplified fragments (13) was produced by OPAF-07. Number of polymorphic bands per primer ranged between 7(OPAE-04 and OPAE-05) and 11(OPAF-07). PIC ranged between 0.18(OPAF-08) and 0.49 (OPAE-05). Dendrogram constructed from the scored bands of the primers presented in Figure 1 shows that they clustered into two groups with intra and inter group variations. Primers OPAD-09, OPAE-04 and OPAF-08 clustered and were differentiated from the rest of the three. Table 4 shows information on occurrence of private allele by which the pectoral spine sub-groups of *C. gariepinus* can be differentiated. Despite similar values of percentage polymorphic band, private alleles were encountered in individuals of both subgroups. However, bands were more polymorphic in the peses group than the non peses (78.00 and 69.84%PB, respectively). Specific homogeneous sites were obtained in 11 cases. All individuals in both groups inherited allele j and k in OPAE-09. However, all loci were heterogeneous in OPAD-09 in peses group while two were homogenous in non peses group. Homogeneity of a particular allele in all members of a group could indicate its suitability as a marker for such group. OPAF-07 was differentially inherited by the two groups; it was uniformly inherited at one site (i) by all individuals in the peses group only. This makes it a potential differentiating site for the phena group. Also, OPAD-09 showed no private allele in peses indicating that the marker is not informative for the category peses but had 2 private alleles in the non-peses thus showing a sub-division or variant in this category. The genetic analysis confirmed the morphological assignation of each *C. gariepinus* groups based on pectoral spine but also highlighted subtle genetic intra-variability. The later being able to give further information on genetic basis of morphologically divergence groups and was able to show within sub-group genetic variability pattern. Dendrogram showing the cluster analysis of the individuals' genotype is presented in Figure 2. The UPGMA cluster diagram identified two major genotypic

**Table 2.** Polymorphic rapid operon primers in *C. gariepinus*: their code, sequence and size range.

S/N	Primer code	Sequence (forward)	Size range (bp)
1	OPAD - 09	TCGCTTCTCC	200 - 3500
2	OPAE - 04	CCAGCACTTC	250 - 2500
3	OPAE - 05	CCTGTCAGTG	150 - 3000
4	OPAE - 09	TGCCACGAGG	200 - 3000
5	OPAF - 07	GGAAAGCGTC	250 - 3000
6	OPAF - 08	CTCTGCCTGA	150 - 3500

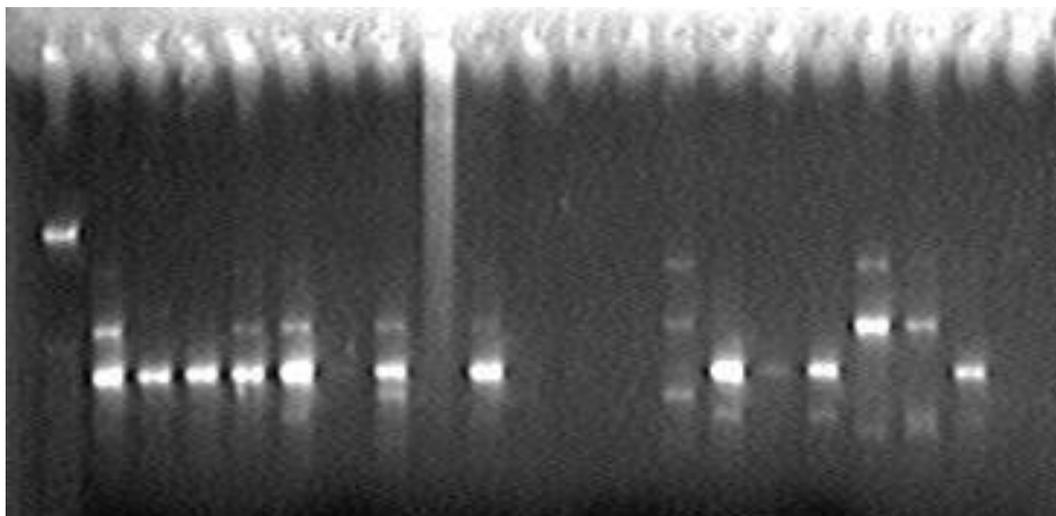
bp, Base pair.

**Table 3.** Primer code, total number of band locus detected (NBL), number of polymorphic band (NPB), average polymorphic band (%PB), polymorphic information content (PIC), private allele per primer (NO. of PA), total number of individual band per primer (NIB) and relative band frequency (Av.BF) generated by the six RAPD primers.

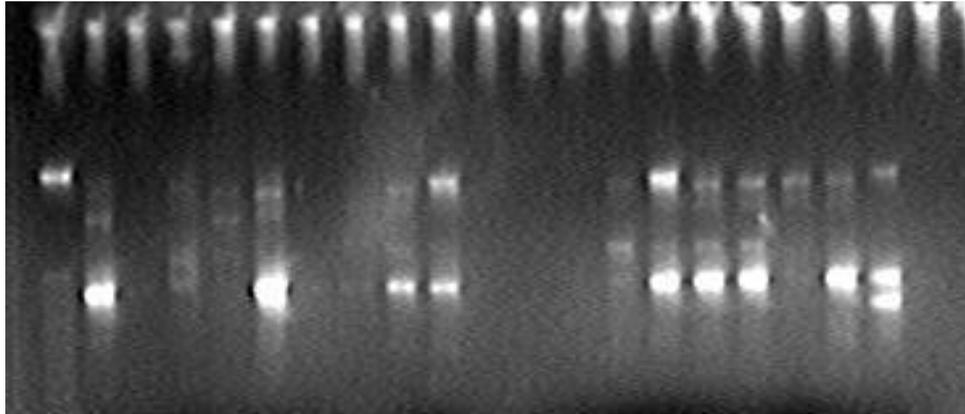
Primer code	NBL	NPB	%PB	PIC	No. of PA	NIB	Av.BF
OPAD - 09	9	8	89.00	0.20	0	82	0.11
OPAE-04	9	7	77.78	0.3457	0	102	0.14
OPAE-09	11	9	81.82	0.2975	0	137	0.18
OPAF-08	10	9	90	0.18	0	104	0.14
OPAE-05	11	7	54.55	0.4959	0	137	0.18
OPAF-07	13	11	76.92	0.3551	1(14)	184	0.25
Total	63	51			1	746	

NBL, Number of band locus; NPB, number of polymorphic band; %PB, percentage polymorphic band; PIC, polymorphic information content; NIB, number of individual band.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

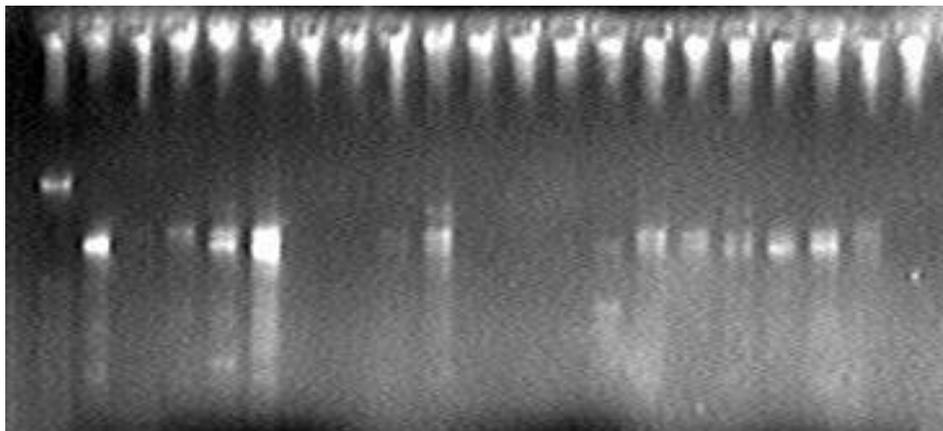
**Plate 1.** Gel phenogram of OPAD - 09

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



**Plate 2.** Gel phenogram of OPAE 04.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



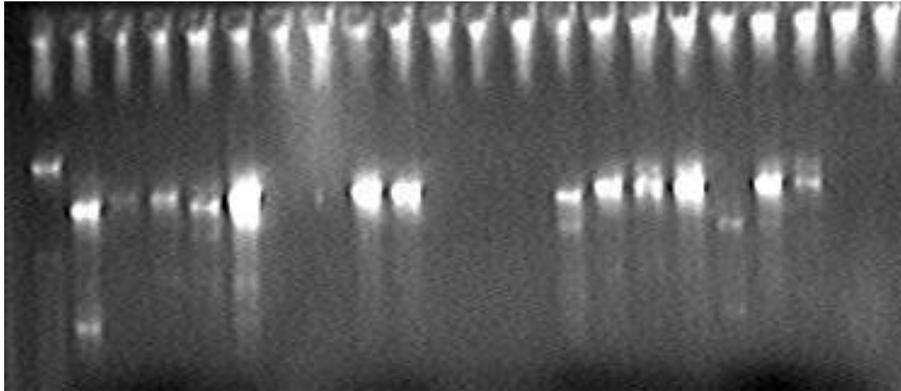
**Plate 3.** Gel phenogram of OPAE 09.

groups with inter and intra group relationships. It also confirmed genetic background for phenotypic separation of the population via pectoral spine; all individuals in the first cluster were from the peses group while all the non peses individuals were on the second cluster. However, all the groups had varied interrelationships showing a highly heterogeneous population. Classification statistics (Table 5) revealed that the initial phenotypic grouping was 100% which is in accordance with the genotypic grouping obtained in this study.

The study reveals that RAPD markers were suitable in establishing polymorphism in the sub-groups of *C. gariepinus*. Genetic disparity between *C. gariepinus* and some other catfishes using molecular tools has been

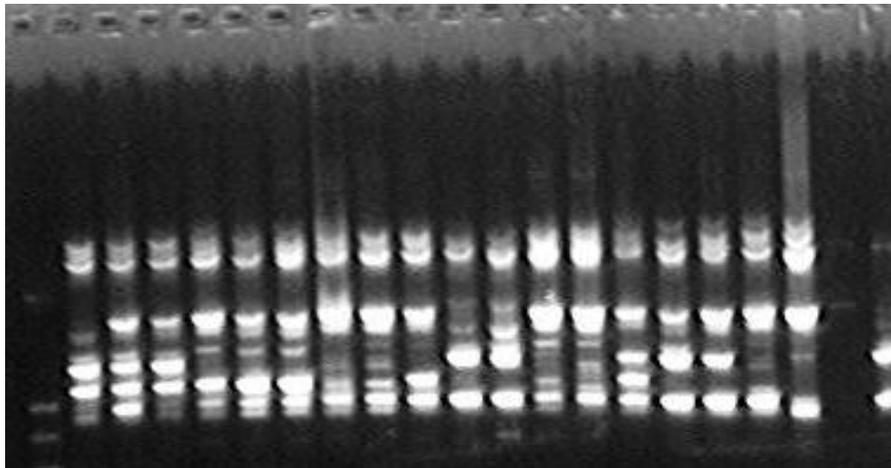
reported by Galbusera et al. (1996), Agnese and Teugels (2001) and Na-Nakorn et al. (2004). Application of the RAPD technique in several fish characterization and genetic variation studies has been reported in Bardakci (2001). The result of the current study indicates that the RAPD-PCR analysis is equally suitable in establishing genetic variability in sub-populations of *C. gariepinus*. The RAPD primers were polymorphic in the population and its sub groups, it also established pattern of intra and inter group variations thus showing the efficiency of the RAPD primer in molecular genetics studies in the populations. It also supported the usefulness of the RAPD primer in genetic studies in *C. gariepinus* as discussed by Saad et al. (2009) and in genetic variability

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



**Plate 4.** Gel phenogram of OPAF -08.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



**Plate 5.** Gel phenogram of OPAE-05.

studies as reported in Almeida and Sodre (2002), Quibai et al. (2006) and Hung et al, (2005).

Knowledge on genetic variation in *genus Clarias* is important as it would facilitate better identification (Teugels et al., 1992; Agnese et al., 1997; Rognon et al., 1998) as well as assist in detection of introgression and hybridization with other species (Billington et al., 1996). Result of the investigation on genetic variability of the studied population revealed variation in both within and between sub-groups of *C. gariepinus*. This indicates that the population expressed genetic heterogeneity. The result thus agreed with the earlier reported observation of heterogeneous phenotypic structure of the population. However, genetic variability of the stock will have to be

maintained in order to sustain the genetic potential of the fishery. This is because of the reported continual decreasing population size of *C. gariepinus* in Asejire dam coupled with expanding pressure on its use for research and mass propagation. Smallness of population in fragmented catchment like the study area will facilitate in-breeding and its attendant depression in the future. However, the variability pattern as documented in this study would be useful in monitoring and maintenance of *C. gariepinus* genetic pool in the catchment. Maintenance of stocks genetic variability would involve minimizing mating of closely related individuals.

Saad et al. (2009) reported that failure to maintain stocks genetic variability in *Oreochromis niloticus* families

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

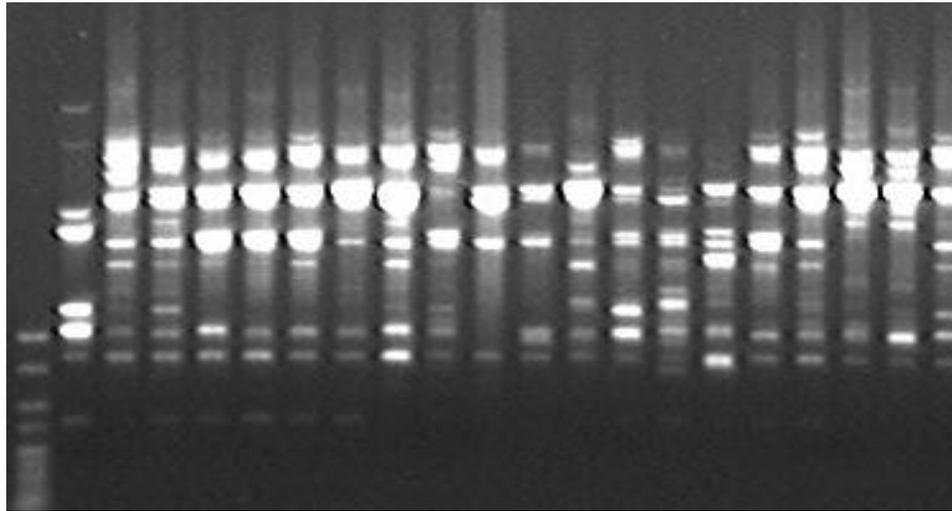


Plate 6. Gel phenogram of OPAF-07.

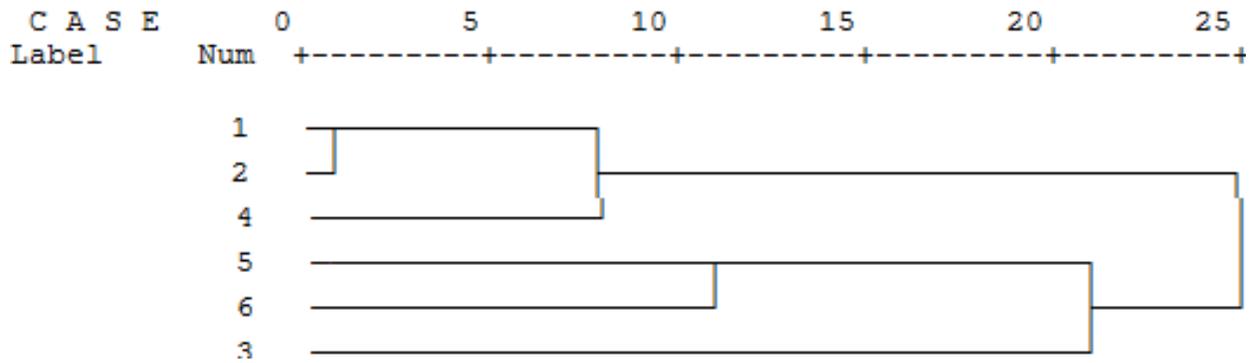
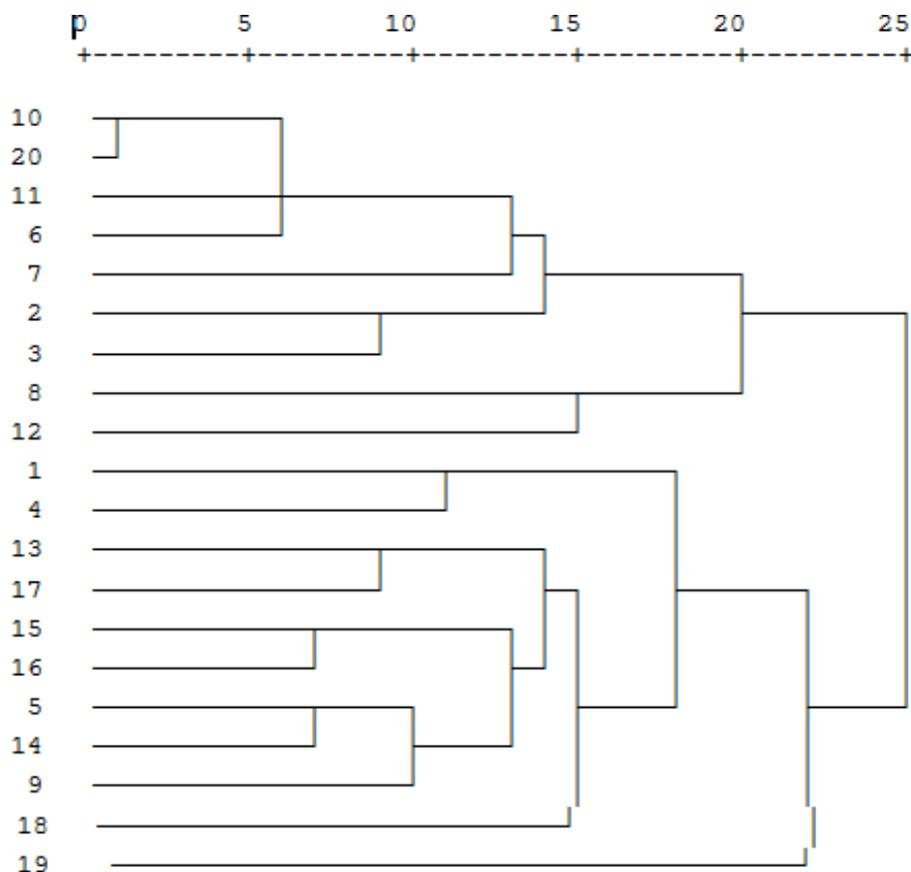


Figure 1. Dendrogram showing average linkage between the six polymorphic primers with respect to the studied population (primers clustered into two groups with intra and inter group variations). 1, OPAD-09; 2, OPAE-04; 3, OPAE-09; 4, OPAF-08; 5, OPAE-05; 6, OPAF-07.

Table 4. Occurrence of private allele by pectoral spine sub-groups of *Clarias gariepinus* after RAPD primers analysis.

Primer code allele	No. of Homogeneous sites		
	Peses	Non-peses	Differentiating
OPAD-09	0	2 (b,l)	2(b,i)
OPAЕ-04	2(h,i)	4 (e,g,h,l)	2(e,g)
OPAЕ-09	2(j,k)	2 (j,k)	-
OPAF-08	1(j)	3(e,g,i)	2(e,g)
OPAЕ-05	6(c,d,g,h,l,j)	6(a,b,c,d,h,i)	4(a,b,g,i)
OPAF-07	3(c,d,i)	2(c,d)	1(j)
Total (MB)	14	19	11
%PB	78	69.84	

\*MB, Monomorphic band; PB, polymorphic band.



**Figure 2.** Dendrogram representing the inferred phylogenetic relationship in *C.gariepinus* population based on RAPD analysis. Individuals 13, 15, 17, 18, and 19 belong to S group while others belong to C group.

**Table 5.** Classification results (a) for the *C. gariepinus* population's genotypes.

Parameter	Score	Predicted group membership		Total
		0.00	1.00	
Original count	0.00	5	0	5
	1.00	0	15	15
%	0.00	100.0	0.0	100.0
	1.00	0.0	100.0	100.0

a = 100.0% of original grouped cases correctly classified.

could be attributed to un-minimized mating of closely related individuals. However, minimizing mating of closely related individuals in the study area may not be feasible going by its observed fragmented structure as observed in an earlier assessment of the catchment and the declining state of *C. gariepinus* stock in the catchment (Omoike, 2004; Oyebola et al. *in press*). Moreover, this will be heightened by the pressure on its fishery as major source of wild brood-stock for research and mass

propagation (FAO, 2012), in hatchery stock improvement coupled with its reproductive versatility (Nukwan et al., 1990) and the sporadic growth of its hatcheries in the region. However, collections from the capture environment could be isolated in special hatchery under breeding programmes and produced through rotational mating selection (RMS) method (PNGS, 2007) but this has to be done under restricted management in order to achieve the desired objective (Saad et al., 2009).

Canonical classification analysis of the genotypic data showed 100% differentiation of the sub-groups genotypes, presence of private alleles and the sub-groups can be differentiated using OPAF-07. These indicate a potential advantage in marker assisted selection for these potential *C. gariepinus* varieties. This observation may have implications apart from taxonomy. Saad et al. (2009) reported that generated RAPD-DNA markers may be associated with DNA regions which affect economic characters. Moreover, earlier study on biochemical differentiation of the sub-groups had revealed a differentiating marker that has nutritional and medical importance. The identified locus in the current study may therefore be confirming that the earlier observations has DNA basis and their differences would be heritable. The UPGMA dendrogram agreed with the phenetic classification of the pectoral spine groups thus indicating that the sub-groups are genetically different. Within population variation was observed in both groups which indicated that the populations were genetically heterogeneous. Earlier phenotypic studies on the population had revealed that *C. gariepinus* population obtained from the Asejire dam was heterogeneous and within sub-groups variation existed. The current result may therefore be confirming that the pattern has genetic basis.

In conclusion, the pectoral spine variants are genetic variants and are potential varieties for *C. gariepinus* while the RAPD primers were suitable genetic markers for establishing variability in the populations. Bowditch et al. (1993) reported that detection of genetic variation is essential to a wide range of comparative genetic research endeavours which include gene mapping, individual identification, parentage determination, population genetics and molecular phylogenetics. The obtained result in this study would therefore have wide application in utilization and management of genetic resources in *C. gariepinus*.

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*Full Length Research Paper*

## Establishment of core collection from apricot germplasm in China

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**This study aimed at establishing a core collection based on the analysis of data from simple sequence repeat (SSR) alleles and morphological and agronomical traits (MOR) of the primary core collection from apricot germplasm resources. The index of genetic diversity, and frequency ratios of retention and loss of the alleles were studied between cluster and random sampling methods at five sampling ratios. The results demonstrate that the cluster sampling method preceded random sampling, and cluster sampling of SSR combined with MOR at the rate of 80% was the best sampling strategy among all the sampling methods. Based on this sampling strategy, 120 accessions were selected as the core collection of apricot, which retained 100% alleles in the primary core collection and 100% phenotypic characters. The core collection developed had also been evaluated by using the data of six quantitative traits, which showed that the established core collection could well represent the genetic diversity of the original collection of 1501 apricot accessions.**

**Key words:** Apricot, core collection, sampling strategy, simple sequence repeat (SSR) molecular marker.

### INTRODUCTION

Apricot is an important fruit crop in the world. Usually, it is consumed fresh or dry, but processing is also favored due to their specific taste, aroma and nutritive values. As the origin center of apricots, China has the richest resources of both wild and cultivated varieties, including 2000 varieties or types (Wang, 1998), which provide considerable opportunities for genetic research and breeding. However, huge numbers of accessions represent challenges for their conservation, evaluation, identification and utilization (Grenier et al., 2000; Tanksley et al., 1997).

Moreover, for perennial woody plants, large plantation area and high cost of management places a severe restriction on their size. So it is more urgent to establish core collection of Chinese apricot resources. The concept

of core collection was initiated by Frankel in 1984, defined as a representative sample of the whole collection with minimum repetitiveness of the genetic diversity of a crop species and its relatives. With the core collection, it is convenient to study and utilize germplasm resources, which has been received by more and more researchers in the entire world. In apricot, we firstly performed the construction of primary core collection from 1501 accession of apricot in China using morphological data (Wang et al., 2011). The size of the primary core collection is still so large that redundancy of some accessions may occur because apricot is usually clonally propagated. Therefore, it is necessary to develop a core collection with the same genetic diversity as the whole collection, but smaller in size than the primary core

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**Abbreviations:** *Ia*, Index of genetic diversity of alleles; *Ip*, index of genetic diversity of phenotype; **MOR**, morphologic and agronomic traits; **RRa**, retained ratio of alleles; **RRp**, retained ratio of phenotype.

collection.

To characterize and evaluate effectively apricot germplasm, molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and inter simple sequence repeat (ISSR) were developed. Among these markers, SSRs are highly polymorphic, informative, co-dominant, technically simple and reproducible, and become common in developing a core collection for some crops (Yao et al., 2008; Zhang et al., 2009a). In addition, when to construct the core collection of a crop, the morphologic data are usually applied extensively because of those data recorded relative comprehensively (Li et al., 2002, 2007; Zhang et al., 2010). However, there were few reports about developing a crop collection using morphologic data combining with SSR data.

The objective of this study was to develop a core collection based on the analysis of Simple Sequence Repeat (SSR) alleles and morphological data (MOR) of the primary core collection of apricot in China, and so as to provide valuable references to the scientific conservation and utilization.

## MATERIALS AND METHODS

The primary core collection of apricot, comprising 150 accessions, was used in this study. This collection was sampled from the initial collection including 1501 accessions of apricot in China and represented 100% of the diversity in the initial collection at the morphological level (Wang et al., 2011).

### Morphological data

Eighteen (18) morphological and agronomical traits were selected in this study. They consist of (1) fruit size, (2) fruit shape, (3) fruit height, (4) lateral width, (5) ventral width, (6) symmetry in ventral view, (7) suture, (8) depth of stalk cavity, (9) shape of apex, (10) ground color, (11) hue of over color, (12) color of flesh, (13) texture of flesh, (14) juice content, (15) soluble solid content, (16) adherence of stone to flesh, (17) ripe stage and (18) flesh fiber content, including 6 quantitative variables and 12 qualitative class variables, which were all related to fruit morphology. These phenotypic characters of about 150 accessions apricot germplasm, collected from the apricot repository of Beijing and Xiongyue, Liaoning province, were recorded for two years using the 57 defined descriptors for apricot by International Union for the Protection of New Varieties of Plants (UPOV) (TG /70/4, 2005) and the book 'descriptors and data standard for apricot (*Prunus armeniaca* Mill)' for observation on fruit fruit (Liu and Liu, 2006), 25 typical fruits were selected from each of the analyzed accession. The material was grafted on apricot seeding rootstocks with at least three replications per genotype.

According to the method of Li et al. (2002), the quantitative characters were quantified into 10 categories, where the distance between two neighboring categories was every 0.5 standard deviation.

### SSR data

Total genomic DNA was extracted from fresh leaf following the

method of Doyle and Doyle (1987). PCR amplification and electrophoresis were performed as described by Wang et al. (2011). The SSR primers (Table 1) were designed based on the nucleotide sequences reported by Testolin et al. (2000), Sosinski et al. (2000), Dirlwanger et al. (2002), Lopes et al. (2002) and Hagen et al. (2004). These 22 pairs of primers were firstly screened for amplification of polymorphic and unambiguous bands in the 150 accessions. An example of amplification products of SSR primer pair (UDP97-401) is shown in Figure 1. Only clear, well defined and reproducible bands were recorded for developing a core collection. Presence (1) and absence (0) of each band was scored. The dataset was converted into a mathematical matrix, which was used to perform statistical analysis and calculate the number of alleles by using Cervus version 2.0 and PopGene version 1.32 softwares. The polymorphic microsatellite markers identified in this study is listed in Table 1. A total of 196 alleles were identified at 22 SSR loci in 150 accessions from the apricot primary core collection. The number of alleles per locus ranged from 5 to 15, with an average of 8.91 alleles per locus.

In the 22 loci, the expected heterozygosity values varied greatly, with an average of 0.731. In addition, the average value of polymorphic information content (PIC) was 0.695, ranging from 0.44 to 0.86.

### Sampling scheme of apricot core collection

A flowchart of the methodology used in the establishment of core collection is presented in Figure 2. Sampling schemes were developed at two levels, that is, the sampling method and sampling proportion. The sampling methods included cluster analysis based on 3 different data (SSR, MOR, SSR + MOR) and random sampling. For the size of core subset under each sampling method, sampling proportion from the primary collection was designed as 50, 60, 70, 80, 90 and 100%. Combining the sampling methods with the sampling proportion, 24 sampling strategies were used to develop core collection, and thus, 24 core collections were generated. In order to avoid losing some important biological types, the sample selecting of the core collection were conducted according to the determined sampling strategies in combination with many other germplasm information at the same time. Some accessions with distinct traits will be deliberately added to the list of the core collection if not being selected.

### Evaluating parameters for sampling methods

Three evaluating parameters were selected according to the study of Li et al. (2002, 2007) with some modification, including index of genetic diversity ( $I$ ) of phenotype (or alleles), retained ratio ( $RR$ ) of phenotype (or alleles), frequency and ratio of loss of alleles. Formulas for  $I$  and  $RR$  are as follows:

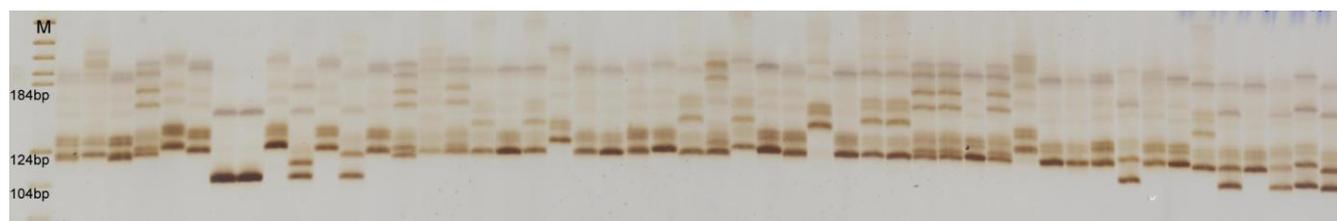
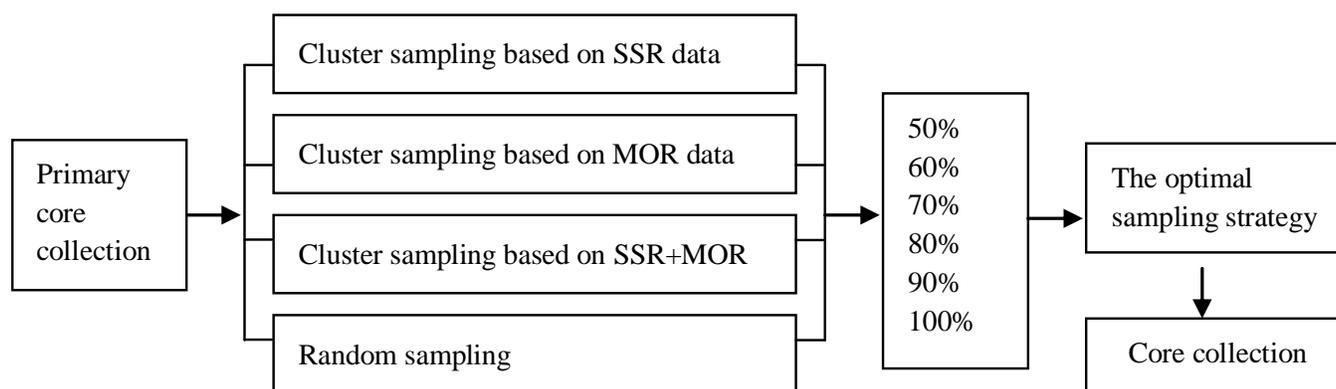
$$I = \frac{-\sum_i \sum_j P_{ij} \ln P_{ij}}{N}, \quad RR = \frac{\sum_i M_i}{\sum_i M_{i0}}$$

Where,  $P_{ij}$  is the frequency of the  $j^{\text{th}}$  phenotype (or alleles) in the  $i^{\text{th}}$  trait (or locus);  $N$  is the total number of traits (loci); where,  $M_{i0}$  is the number of the  $i^{\text{th}}$  phenotype (or alleles) of the initial germplasm group;  $M_i$  is the number of the  $i^{\text{th}}$  phenotype (or alleles) of core subset.

The loss frequency of alleles ( $P$ ) was divided into two ladders, that is  $P \leq 0.01$  and  $0.01 < P \leq 0.03$ ; the loss ratio of alleles at different  $P$  range = the lost number of alleles in one core subset / the lost

**Table 1.** Polymorphic microsatellite markers identified in the construction of apricot core collection.

Locus	Ogrin	Repeat motif	No. of alleles	PIC	Heterozygosity
UDP96-005	<i>P. persica</i>	(AC) <sub>16</sub> TG(CT) <sub>2</sub> CA(CT) <sub>11</sub>	9	0.648	0.694
UDP97-401	<i>P. persica</i>	(GA) <sub>19</sub>	10	0.638	0.680
UDP97-402	<i>P. persica</i>	(AG) <sub>17</sub>	7	0.700	0.741
UDP98-406	<i>P. persica</i>	(AG) <sub>15</sub>	11	0.848	0.866
UDP98-409	<i>P. persica</i>	(AG) <sub>19</sub>	9	0.768	0.800
UDP98-411	<i>P. persica</i>	(TC) <sub>16</sub>	11	0.810	0.834
UDP98-412	<i>P. persica</i>	(AG) <sub>28</sub>	9	0.829	0.849
Pchcms4	<i>P. persica</i>	(CA) <sub>9</sub>	6	0.440	0.470
Pchgms4	<i>P. persica</i>	(CT) <sub>21</sub>	7	0.549	0.624
Pchgms10	<i>P. persica</i>	T <sub>19</sub> A <sub>10</sub>	6	0.480	0.562
BPPCT001	<i>P. persica</i>	(GA) <sub>27</sub>	6	0.567	0.612
BPPCT002	<i>P. persica</i>	(AG) <sub>25</sub>	8	0.797	0.825
BPPCT012	<i>P. persica</i>	(CT) <sub>13</sub> CC(CT) <sub>7</sub>	11	0.684	0.706
BPPCT028	<i>P. persica</i>	(TC) <sub>15</sub>	5	0.525	0.606
BPPCT029	<i>P. persica</i>	(GA) <sub>12</sub> (CAGA) <sub>4</sub>	10	0.776	0.800
BPPCT030	<i>P. persica</i>	(AG) <sub>25</sub>	5	0.476	0.512
ssrPaCITA15	<i>P. armeniaca</i>	(TC) <sub>15</sub>	10	0.787	0.814
ssrPaCITA19	<i>P. armeniaca</i>	(TC) <sub>16</sub>	15	0.860	0.876
AMPA095	<i>P. armeniaca</i>	(AC) <sub>13</sub> T(AC) <sub>4</sub>	13	0.674	0.694
AMPA105	<i>P. armeniaca</i>	(AG) <sub>11</sub>	11	0.843	0.862
AMPA109	<i>P. armeniaca</i>	(TG) <sub>11</sub> (AG) <sub>9</sub>	8	0.780	0.810
AMPA112	<i>P. armeniaca</i>	(AG) <sub>18</sub>	9	0.814	0.838
Average			8.91	0.695	0.731

**Figure 1.** An example of SSR primer pair (UDP97-401) amplification patterns in some apricot accessions (M: Marker).**Figure 2.** Method for establishment for the core collection of apricot cultivars.

number of alleles in the primary core collection, the  $R$  value of one trait in the initial collection.

### Evaluation of the representation of core collection based on the validated sampling methods

To determine the representation of the core collection, 6 quantitative traits and 6 evaluating parameters were selected to compare the entire and core collection. The 6 traits include fruit weight, fruit height, lateral width, ventral width, soluble solid content and ripening time. The 6 evaluating parameters are maximum, minimum, range ( $R$ ), coefficient of variation ( $CV$ ), variance of phenotype value ( $VPV$ ) and ratio of trait retained ( $RTR$ ). The values of  $R$ ,  $CV$ ,  $VPV$  and  $RTR$  were calculated using the following formulas, respectively.

$R$  = the maximum value of one trait – the minimum value of one trait:

$$CV = \frac{\sum \sqrt{\frac{\sum (X_{ij} - \bar{X}_i)^2}{n-1}}}{\bar{X}_i} \times 100$$

Where,  $X_{ij}$  is the phenotypic value of the  $j^{\text{th}}$  accession in the  $i^{\text{th}}$  trait,  $\bar{X}_i$  is the mean of phenotypic values of all accessions in the  $i^{\text{th}}$  trait,  $n$  is the number of accessions,  $N$  is the total number of traits.

$$VPV = \frac{\sum \left[ \frac{STD_i \sum_j (X_{ij} - \bar{X}_i)^2}{M_i - 1} \right]}{N}$$

Where,  $STD_i$  is the standardization for the  $i^{\text{th}}$  trait;  $X_{ij}$  is the phenotypic value of the  $j^{\text{th}}$  accession in the  $i^{\text{th}}$  trait;  $\bar{X}_i$  is the mean of phenotypic values of all accessions in the  $i^{\text{th}}$  trait,  $M_i$  is the number of the  $i^{\text{th}}$  phenotype in core collection,  $N$  is the total number of traits.

$$RTR (\%) = \frac{\text{The } R \text{ value of one trait in the core subset}}{\text{The } R \text{ value of one trait in the initial collection}} \times 100$$

The values of  $I$  was calculated according to the aforementioned formulas.

## RESULTS

### The analysis of the index of genetic diversity among four sampling methods at different sampling proportion

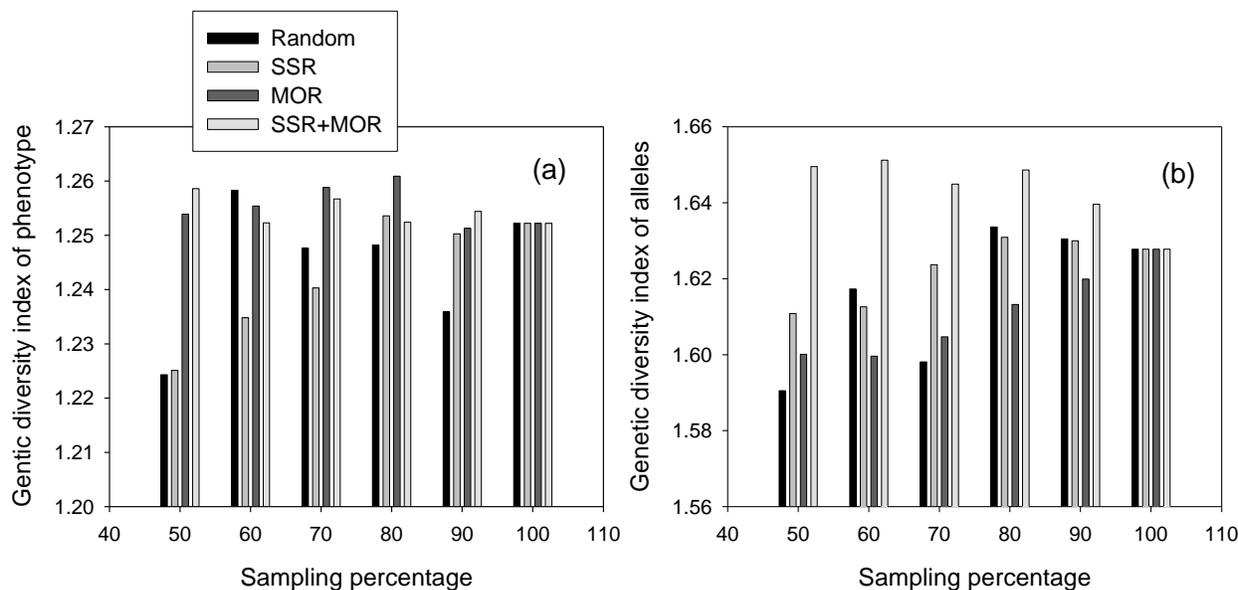
For the index of genetic diversity of phenotype ( $Ip$ ), as

shown in Figure 3a, when randomly sampling methods were used, there was no regularity in the changes of the  $Ip$  values among different sampling proportion. When cluster sampling was based on SSR + MOR, the  $Ip$  value reached the most at the sampling percentage of 50%, and then slightly decreased with the sampling percentage increased. Whereas, for other two cluster sampling methods, the  $Ip$  value presented an increasing trend with the sampling ratio increased. At the same sampling ratio of 60, 70 and 80%, the  $Ip$  values in the core collection using cluster sampling methods based on MOR was higher than that using other two cluster sampling methods. It can be seen from Figure 3b, for the index of genetic diversity of alleles ( $Ia$ ), it was also no regularity in the changes of the  $Ia$  values when randomly sampling method was used to construct the core collection. At any sampling ratio point, the  $Ia$  value of the core collection using cluster sampling methods based on MOR was all lower than that using other two cluster sampling methods. Whereas, the highest level occurred in the cluster sampling methods based on SSR + MOR. For the cluster sampling methods based on SSR, the  $Ia$  values of alleles were increased with the sampling ratio increased, and reached the highest at the sampling percentage of 80%.

Comparing Figure 3a with 3b, it can be seen that the value of the index of genetic diversity in allele was obviously higher than that in phenotype at the same sampling ratio. Under most sampling ratios, the  $Ip$  values were the highest when using cluster sampling methods based on MOR, followed by SSR + MOR, the lowest was SSR. However, for the  $Ia$  value, it reached the highest when using cluster sampling methods based on SSR + MOR. The value of the index of genetic diversity for one core collection is higher, represented the core collection is more perfect. All together, the cluster sampling methods based on SSR + MOR was more preferred than the other three sampling methods.

### The analysis of retained ratios among four sampling methods

The retained ratios of phenotype ( $RRp$ ) were analyzed and compared among the 24 core collections established according to different sampling strategies (Figure 4a). Similar to the index of genetic diversity, when randomly sampling methods were used, there were no regular changes in  $RRp$  values among different sampling proportion. For the three cluster sampling methods, the  $RRp$  values of the core collections rapidly increased with the sampling percentage increased. At any sampling percentage, the  $RRp$  values of the core collection constructed using cluster sampling based on MOR was maximum among the four sampling methods, and at sampling percentage of 60%, the core collection was able to preserve 100% phenotype of the primary collection. Comparing MOR + SSR with SSR, the former methods was relatively suitable; the  $RRp$  values reached higher to



**Figure 3.** Influence of different sampling methods on genetic diversity index of core collections.

98.39% at sampling percentage of 50%. Whereas, at the same sampling ratio, the  $RRp$  values was only 96.77% when using cluster sampling based on SSR. A total of 22 pairs of SSR primers were selected for amplification of high polymorphic and unambiguous bands. A total of 196 alleles were identified at 22 SSR loci in 150 accessions from the apricot primary core collection. Statistically analyzed the number and the frequency of the alleles of the core collections using different sampling strategies, and calculated the retained ratios of alleles ( $RRa$ ). As presented in Figure 4b, for the four sampling methods, the  $RRa$  values of the core collections almost increased with the sampling percentage increased. The  $RRa$  values of the core collection constructed using cluster sampling based on MOR + SSR was obviously higher than that using other three sampling methods at corresponding sampling percentage.

Comparing Figure 4a with 4b, it can be seen that the  $RRp$  values was higher than the  $RRa$  values at the same sampling ratio among the four sampling methods. For example, at sampling percentage of 50%, the  $RRp$  values were higher than 96%; whereas, the  $RRa$  values were only 92%. In addition, under most sampling ratios, the  $RRp$  and  $RRa$  values of the core collections constructed using cluster sampling based on MOR + SSR all remained higher than the other three sampling methods.

#### Analysis of the loss of alleles among four sampling methods

The objective of the development of core collection is to select a representative sample of the whole collection

with minimum repetitiveness and maximum genetic diversity of a crop species and its relatives. During the process of constructing the core collection, it is inevitable that the loss of alleles will occur when the genetic repetitiveness was eliminated. The lower frequency allele was usually easy being lost during the sampling compressed. The loss ratio of alleles at different frequency range among different sampling strategies was analyzed in the present study. The result is shown in Figure 5. At similar sampling percentage, the loss ratio of alleles was highest when  $P \leq 0.01$  among the four sampling methods. Where, the  $P$  is the frequency of a lost allele presented in the primary core collection. Comparatively, the loss ratio of alleles was much lower when  $0.01 < P \leq 0.03$ , and all the alleles of the primary core collection would remained in the core collection when  $0.03 < P \leq 0.06$ .

As shown in Figure 5a, the alleles of  $P \leq 0.01$  were lost in various degrees among the four sampling methods. At the sampling percentage from 50 to 70%, for the three cluster sampling methods, the loss ratio of alleles decreased as the sampling percentage increased. The loss ratio of alleles in the core collection constructed using cluster sampling based on MOR was the highest than that using other three sampling methods. According to this method, the loss ratio of alleles was still higher than 0.27 even if the sampling percentage increased higher to 80%. Correspondingly, the loss ratio of alleles was all lower than 0.17 for the other three sampling methods.

The loss ratio of alleles was much lower of  $0.01 < P \leq 0.03$  than that of  $P \leq 0.01$ . As the sampling percentage increased, the loss ratios of alleles were all rapidly decreased among all the sampling methods

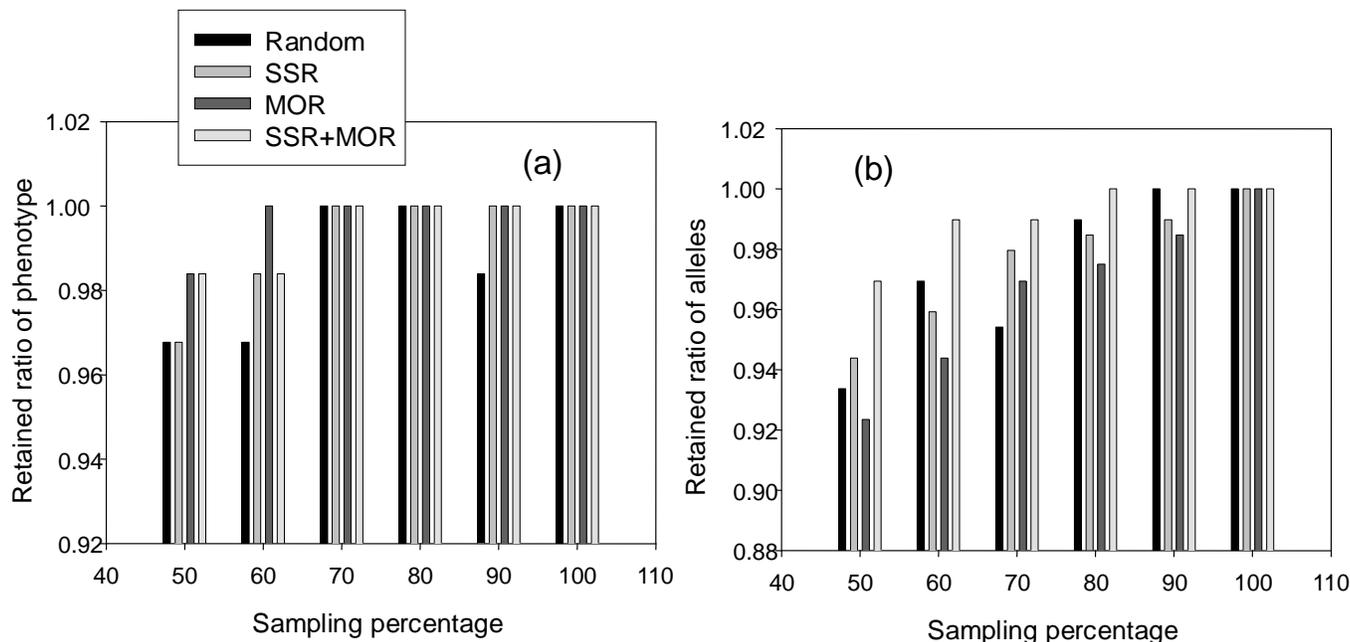


Figure 4. Influence of different sampling methods on retained ratio of core collections.

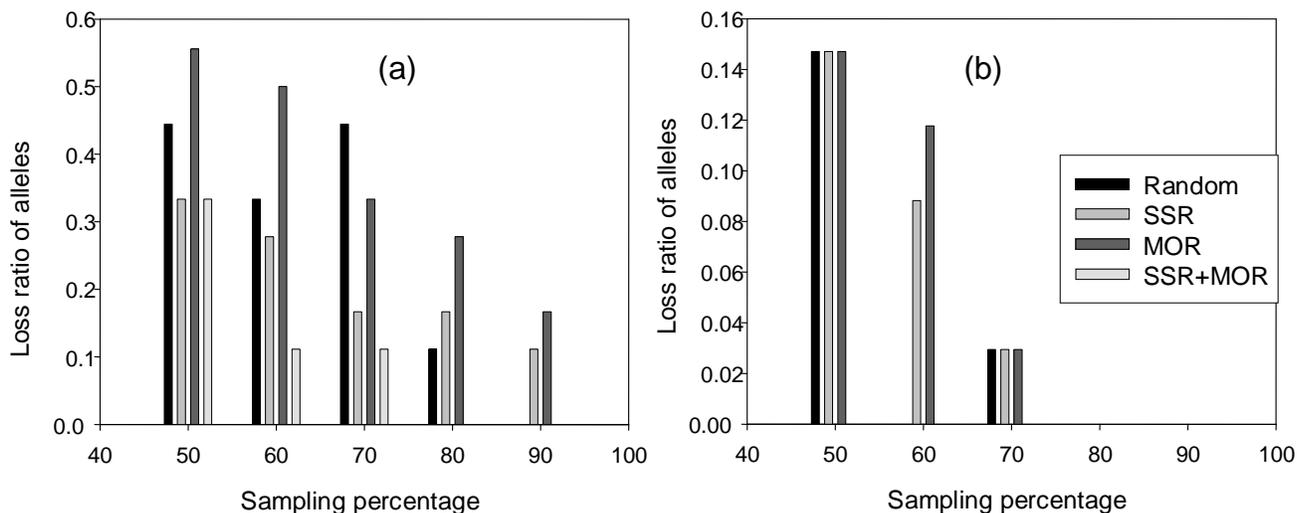


Figure 5. Analysis of ratio of alleles lost under different sampling methods. (a)  $P \leq 0.01$  (b)  $0.01 < P \leq 0.03$ .

(Figure 5b).

These results indicate that it was an effective way to retain these alleles of  $0.01 < P \leq 0.03$  by increasing the sampling percentage. At the same sampling percentage, the loss ratio of alleles of the core collection constructed using cluster sampling based on SSR + MOR was the most low, and the value was decreased to zero at sampling percentage of 50%. When sampling percentage increased to 80%, the loss ratio values were also decreased to zero. For the alleles of  $0.03 < P \leq 0.06$ , the

loss ratio values were all zero among all the sampling strategies, that is, all the alleles of  $0.03 < P \leq 0.06$  all remained in the core collection constructed using any sampling methods. Above these results, it was difficult to retain the alleles of the lower  $P$  value, even if the sampling percentage increased. However, for the alleles of the higher  $P$  value, the loss ratios would be rapidly decreased with the sampling percentage increased. Thus, it is an effectively way to retain these alleles of the higher  $P$  value by increasing the sampling percentage.

## Determination of the apricot core collection

According to the analysis of the 24 core collections established based on 24 sampling strategies, it is suggested that the optimal sampling methods were cluster sampling based on SSR + MOR, and the suitable sampling percentage was 80%. Using this sampling strategy, we have established the apricot core collection with 120 accessions, including 75 accessions for flesh market and processing, 11 accessions for kernel consuming, 7 accessions for ornamental cultivars, 7 accessions for newly bred cultivars (or lines), 13 accessions for introduced germplasm and 7 accessions for inter-specific hybrids. The name of germplasm and their characteristics are listed in Table 2.

The core collection remained both 100% alleles of the primary core collection and all the phenotype traits of the initial collections, and the valuable germplasm with different economic traits (for example, fruit size, fruit quality, early ripeness, self fertility, disease tolerance, cold resistance, etc.) were all remained in the core collection. For example, 'Liquanerzhuanzi' is one apricot cultivar of large fruit size, the soluble solids content of fruit in 'Keziximixi' is high to 21% and its Vc value was also high to 22.1 mg/100 g FW, 'Anjiana' is a cultivar of high sugar and the content was high to 9.9%, '80A03' is a genotype with very large kernel and the average weight of kernel is high to 0.98, 'Luotuohuang' is an early ripening cultivar (fruit development period is 55 days), 'Kaite' (originally name is Katy) is a cultivar with high ratio of self-fertility (Cao et al., 2012), and the blossom characteristics is special such as 'Liaomeixing' with multiplied petal and 'Lvshanxing' with green sepal, and so on.

## Estimation of the apricot core collection

Five parameters of 6 quantitative traits from the core collection were compared with those of same parameters from the primary and initial collection, and the results are listed in Table 3. Compared with the primary core collection, except soluble solid content, the values of the three parameters (maximum, minimum and range) of other five quantitative traits in the core collection were all same as that in the primary core collection. The CV and VPV values of five traits (except fruit ventral width) were higher in the core collection than those in primary core collection, indicating that the core collection eliminated some genetic redundancy and can represent genetic diversity of the primary core collection very well.

In comparison with initial collection, the CV and VPV values of soluble solid content were lower in the core collection than those in initial collection; whereas, the same two parameters of the other five traits in the core collection were all higher than those in the initial collection, implying that the primary core collection has

retained a large variation in the initial collection. For the retained ratios, there existed some differences among the six traits. The retained ratio of fruit weight was the highest, with a value of 99.69%; fruit lateral width was in the second place with a value of 97.96%, followed by fruit height (80.00%), ripe stage of fruit (78.57%) and fruit ventral width (70.18%). The value of the soluble solid content was comparably lower; however, the value is still high to 64.35%. These results demonstrated that a large variation exists among the 120 apricot accessions at phenotypic level, and the genetic variation in the core collection can well represent the initial collections, though some accessions were lost in the core collection.

All together, these results confirmed that the sampling strategy we selected was well suitable, and the core collection retained all genetic information of the primary core collection or initial collection, and can represent genetic diversity of the initial collection in apricot.

## DISCUSSION

### The data used to construct core collection

Usually, there are three types of data which were used to establish a crop core collection; that is, passport, characteristic and evaluation data (Brown, 1989). The passport data include some information about the collection site, the eco-physiological condition of origin site, breeding background, principles of taxonomy system, etc; the characteristic data represents the characters of the collection, and includes morphological data, biochemistry data, molecular markers, and so on; the evaluation data includes some agronomic traits about yield, quality, stress resistance, and so on.

When constructing the core collection of a crop, the passport data are usually applied extensively, because those data recorded relative comprehensively, and become the most effective data when combining with other data. As one of the criteria used to study core collection, the morphologic data have some virtues, that is, the method of obtaining data is simple and costs less. However, the morphologic traits usually do not reflect the genetic nature because of some interference caused by the environment or man-made conditions. For example, the plant growth and fruit quality of strawberry were all affected by the temperatures after bloom affect plant growth and fruit quality of strawberry (Wang and Camp, 2000). Alcobendas et al. (2012) reported that exposure to sunlight strongly influenced fruit size, weight and skin color of peach.

Thus, it is important to select the morphologic traits with relative stability to evaluate the germplasm resources. Generally, molecular markers can directly reflect the change of genetic germplasm at DNA level, without interference from the environment, and became the valuable data to evaluate the genetic diversity. It was

**Table 2.** List of 120 accessions and their special characteristics in apricot core collection.

No.	Germplasm name	Major trait	No.	Germplasm name	Major trait
1	606Xin	Cold resistance, high acid	2	631Xing	High soluble solids, cold resistance
3	Anjiana	Flesh with high sugar, cold resistance	4	Badouxing	High productivity
5	Baihuwaina	Early ripening, high soluble solids	6	Bairenxing	Ground color of fruit skin is white
7	Beianhedahuangxing	Large fruit size	8	Bianganxing	Early ripening, flesh is firm
9	Cangjiaxing	Firm and sour flesh	10	Caotanmeixing	Flesh is firm
11	Chuanling	Color on sunny side of one-year-old shoot is red brown	12	Chuanzhongxing	High productivity, flesh is firm, high cold and salt resistance
13	Dabaixing	Flesh with high sugar	14	Dafengxing	Large fruit size, high productivity
15	Daofuxing	Wild type, high acid	16	Dapiantouxing	Fruit in ventral view is clearly asymmetric, fruit scab disease resistance
17	Dayexing	Attractiveness (1/3 to 1/2 red blush)	18	Dongning2hao	Texture of flesh is coarse
19	Eezhuanzi	Fruit size is very large	20	Fakuhebao	The productivity is very high
21	Fanglingdaxing	Time of fruit maturity is early, fruit size is large	22	Guanlaoyelianxing	Light red blush
23	Guduxing	Wet and hot climate adaptation	24	Gushandaxingmei	Early fruit maturity, large fruit size
25	Guzanxing	Flesh with high soluble solids	26	Heiyexing	Flesh with high sugar
27	Honglianxing	Purple blush	28	Honghuomeizi	Deep-red blush, firm flesh
29	Hongjinzhen	Strong tree vigor, high productivity, very large fruit size	30	Hongyuxing	Very large fruit size, sour flesh, broad leaf blade
31	Huayinxing	Very large fruit size, high productivity	32	Huluxing	Processing suitability
33	Kailidaxing	Very large fruit size, good postharvest characters	34	Kailixiaoxing	High productivity
35	Kangding2hao	High acid	36	Kezierkumaiti	High soluble solids, high sugar
37	Keziximixi	High soluble solids, for drying market	38	Kuche3hao	Very high productivity, very small fruit
39	Kurenhuangkouwai	More dehiscent fruit	40	Lajiaoxing	Novel fruit shape (similar to capsicum in shape)
41	Lintongyinxing	High productivity, large fruit size, high soluble solids	42	Luotuohuangxing	Combining very early-ripening with superior fruit quality (size, firmness, sugar)
43	Maizihuang	Very early ripening	44	Meitaoxing	Mediated fruit maturity cultivar
45	Niujiaohuang	Medium fruit size	46	Panxiandashaxing	large fruit size, late fruit maturity
47	Qingmisha	Very high soluble solids, degree of branching is weak	48	Ruanhexing	Degenerate stone
49	Shaxing1hao	large fruit size, very late fruit maturity	50	Shipianhuang	Strong apricot aroma
51	Shuangrenxing	Very large fruit size, good postharvest characters	52	Suanmeizixing	Small fruit, processing cultivar
53	Tianedan	Very high soluble solids	54	Tianrenhuangkouwai	Very high productivity, large fruit size with 1/2 red blush over the skin
55	Wangjiaxing	Large kernel, pest and disease resistance	56	Wanshuxing	Very early fruit maturity, good postharvest characters
57	Xinjiangshaxing	large fruit size	58	Xinshuixing	Good postharvest characters
59	Xupuxing	Rootstock cultivar	60	Yangjiyuanxing	Spreading tree habit, very high productivity
61	Yinchuantaoxing	Medium fruit size	62	Yinghong1hao	Fruit with high pectin
63	Youyibaixing	Cold resistance	64	Youyidaxing	Very high cold resistance, high productivity
65	Youyiwumingxing	Very high cold and drought resistance, high productivity	66	Youyixingmei	High acid, high salt tolerance
67	Youyiyinbai	Very high cold resistance, high productivity, white petal some with six petals	68	Zaohuang	Very high cold resistance, very small fruit size, some blossom with six petals
69	Zhanggongyuan	Very high productivity, large fruit with large stone, good postharvest characters	70	Zhoujiaxing	Flesh with high sugar
71	Zhupishuixing	Thicker fruit skin, with strong apricot aroma	72	Zhuyaoxing	High productivity, firm flesh
73	Zhuyaozixing	Large fruit size, fruit-setting alternate year	74	Chaoxianbaixing	Very large fruit size
75	Zaoxing	Early fruit maturity, large fruit size, good postharvest characters	76	Ningxiataoxing	High fruit quality (large size, succulent flesh with high sugar)
77	80A03	Very large kernel, fruit scab disease resistance	78	Baiyubian	Barren soil tolerance
79	Ganke	For fresh market and kernel consuming	80	Huangjianzui	High productivity, medium kernel

Table 2. contd.

No	Germplasm name	Major traits	No	Germplasm name	Major traits
81	Huzhuazi	Cold resistance, high productivity	82	Jiudaomei	Cultivar for flesh market, processing and kernel consuming, fruit-setting alternate year
83	Kelala	High and regular productivity, kernel is large and sweet	84	Longwangmao	Very large kernel
85	Shanxing	Rootstock cultivar	86	Yiwofeng	High productivity
87	Youyi	High cold resistance, fruit-setting alternate year	88	Beilüeshanxing	Green sepal
89	Chongbanshanxing	Flower with multiple petal	90	Chuizhixing	Drooping shoots, narrow leaf blade width
91	Dayuxingmei	Shoot without thorn, big flower	92	Liaomeixing	Flower with multiple petal, very high cold resistance, wild variation of Siberia
93	Meirenmei	Purple red leaf, interspecific hybridization between cherry and plum	94	Yanxingmei	Shoot thorn
95	Hongfeng	Very early fruit maturity	96	Laoshanhongxing	Flesh with high soluble solids, and high sugar, apricot aphid resistance
97	Longken1hao	Very high cold resistance, high pest and disease resistance	98	Longken3hao	High cold resistance, high productivity
99	Longken5hao	Late ripening, good postharvest characters	100	Shiguanzaohong1hao	Very early ripening, self fertility
101	Xinong25hao	Relative area of over color is large, with red blush, attractiveness, late fruit maturity	102	Huiyangbaixing	Fruit scab disease resistance
103	Jifu	Very high cold resistance	104	Jintaiyang	Very early fruit maturity, self fertility
105	Kaitexing	Very large fruit size, self fertility	106	Kaninuo	Small fruit size, sweet-sour flesh with bitter aftertaste
107	Maonaoxing	Self fertility, flesh with high sugar, good postharvest characters	108	Pinghexing	Very small fruit
109	Xinzhoudashi	Very early fruit maturity, tree habit is spreading	110	Zaoju	Specific fruit shape (oblique rhombic)
111	Yidalixing	Flesh with high sugar	112	Meiwuming	High fruit quality (attractiveness, size, firmness, sugar)
113	Jinfu	High productivity, processing ability	114	Changlixingmei	Sour flesh
115	Hongmeixing	Ground color of skin is red, red flesh	116	Jinhuangxingmei	Large fruit size, high productivity, good postharvest characters
117	Limixing	Late-blooming cultivar	118	Longyuanhuangxing	Dwarf tree stature, very high cold resistance
119	Meiguolixing	Dwarf tree stature, late-blooming	120	Qianxianmeixing	High pest and disease resistance, high productivity

was an effective validating method to investigate the alleles presented in the initial collection whether if also presented in the core collection (Gao et al., 2005). Zhang et al. (2011) constructed the core collection of mulberry using the data of ISSR molecular marker. Whereas, as to the present technology, it may be still labor-intensive and time-consuming to evaluate all the genetic germplasm of one species directly using molecular method (Dong et al., 2003; Zhang et al., 2009a) when the number of crop germplasm is huge.

He et al. (2002) proposed that it is an effective method to construct core collection by morphological data combining molecular marker data. In this study, the core collection established by using different data was evaluated and demonstrated that the core collection based on

the single type of data had lower genetic diversity and lower remained ratio. Whereas, the core collection constructed by combining the morphological and agronomical traits data with the molecular marker data had good representative of the initial genetic resources of apricots (Figures 3 and 4). The probably reason was as follows: If only based on the SSR data or other molecular data to construct the core collection, some important germplasms may be lost when the SSR loci selected in the study was not many. Similarly, if only using the morphological data, some germplasms also may be lost because of some interference caused by the environment or man-made conditions.

This study provides some powerful evidence. The best sampling strategy was the cluster sampling method using SSR data combined with

MOR data at the rate of 80% among all the sampling methods in this study.

Based on this sampling strategy, in combination with many other germplasm information such as productivity, stress resistance and fruit quality at the same time, 120 accessions were selected as the core collection of apricot (Table 2), which retained 100% alleles in the primary core collection and 100% phenotypical characters, well represented the genetic variance of the primary core collection and the initial collection. Thus, the use of all available information (characteristic and passport data) was found to be valuable for the establishment of apricot core collection. This result was similar to the reports from Diwan in the annual *Medicago* species, which used valuation and passport data to assemble the core collection (Diwan et al., 1995).

**Table 3.** Evaluation of core collection in apricot.

Parameter		Fruit weight (g)	Soluble solid content (%)	Fruit development days (d)	Fruit height (cm)	Lateral width (cm)	Ventral width (cm)
Original germplasm	Maximum	133.0	29.0	190	7.2	6.7	6.6
	Minimum	2.7	6.0	50	1.7	1.0	1.7
	Range	130.3	23	140	5.5	5.7	4.9
	CV	44.48	20.91	15.12	15.72	15.62	16.11
	VPV	318.18	7.50	139.01	0.40	0.35	0.42
Primary core collection	Maximum	133.0	23.5	160	6.3	5.5	6.6
	Minimum	3.1	7.3	50	1.9	1.5	1.8
	Range	129.9	16.2	110	4.4	4.0	4.8
	CV	51.82	17.02	15.02	17.71	20.07	19.32
	VPV	457.54	4.56	138.87	0.51	0.57	0.76
	Retained ratio (%)	99.69	70.44	78.57	80.00	70.18	97.96
Core collection	Maximum	133.0	22.5	160	6.3	5.5	6.6
	Minimum	3.1	7.7	50	1.9	1.5	1.8
	Range	129.9	14.8	110	4.4	4.0	4.8
	CV	53.77	17.96	15.25	18.81	20.61	18.83
	VPV	490.74	5.12	145.06	0.57	0.61	0.57
	Retained ratio (%)	99.69	64.35	78.57	80.00	70.18	97.96

### The size of core collection and sampling strategy

It is another crucial issue to choose and deal with the suitable data and then decision of the sampling strategy when developing a crop core collection, in detailed, including which sampling methods and which sampling proportion can be used to select the core accessions satisfied the requirements from all the initial accessions. Brown (1989) suggested that about 5 to 10% sample size of the entire collection with an upper limit of 3,000 per species would effectively retain about 70% of the alleles of the entire collection. However, in the study of the core collection of annual *Medicago* species, Diwan proposed that the 5 and 10% sample size core collection were judged insu-

fficient to represent the germplasm collection, probably because the annual *Medicago* species germplasm collection contains many species with very few accessions (Diwan et al., 1995).

The previous study results from many crops suggested that the suitable sampling size of the core accessions was usually about 10 to 30% of the entire collections (Li et al., 2002, 2007; Upadhyaya et al., 2008; Zhang et al., 2009b, 2010). Thus, it is considering that the decision of sampling size should be accorded to the genetic structure and genetic diversity; whereas, could not be uniform simply because the number is different among various crop species, and some special characters occurred with the evolution and the intervening from man-made selection to one crop species. In this study, the size of the core

collection (including 120 accessions) was 8% of the initial collection (including 1501 accessions).

For one plant resource, one type of accessions probably many or less, leading to the imbalance in genetic diversity among various accessions. Moreover, there was difference in emphasizing particularly on some genetic structure or genetic diversity from different study departments. As the asymmetric distribution of genetic diversity and the different repetition number of various alleles among the entire accessions, it is necessary to use better sampling strategy to select the core accessions. Based on the sampling strategy, the genetic variance remained as high as possible, and still could not change the genetic structure of the initial collection. According to present literatures, it was optimal to use stratified cluster

sampling methods (Hu et al., 2000; Jansen and van Hintum, 2007; Zhang et al., 2009b). Li et al. (2002) conducted the study on sampling schemes for the establishment of core collection of rice landraces in Yunnan and suggested that clustering sampling methods was invariably better than non-clustering, whenever at the same grouping principles or at the same sampling proportion within group. Similar to this result, clustering sampling methods is much better than random sampling in this study, and cluster sampling of SSR combined with MOR at the rate of 80% was the best sampling strategy used to construct apricot core collection. There were few reports about the assemblage of core collection in horticultural crop species especially in fruit plants germplasm, and thus, there were less available methods. In this study, the molecular marker data was first used to establish apricot core collection, 120 accessions was selected from 1501 initial collections, which enable the utilization to be much convenient. However, as apricot species is a woody plant, it is necessary to validate the practicality of the core collection by growing judge.

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## Full Length Research Paper

# Effects of coagulating enzyme types (commercial calf rennet, *Aspergillus niger* var. *awamori* as recombinant chymosin and *Rhizomucor miehei* as microbial rennet) on the chemical and sensory characteristics of white pickled cheese

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The possibilities of using recombinant chymosin as an alternative coagulant to commercial calf rennet in the production of white pickled cheese was investigated. For this purpose, white pickled cheese produced by using commercial calf rennet, recombinant chymosin (*Aspergillus niger* var. *awamori*) and microbial rennet (*Rhizomucor miehei*) were compared in terms of their chemical and organoleptic properties. The cheese samples were stored in brine containing 12% salt at  $4\pm 1^\circ\text{C}$  for 60 days. In the study, which was carried out in duplicate, pH, titratable acidity, dry matter, fat, fat-in-dry matter, protein, salt, nitrogenous compounds (water soluble nitrogen (WSN), ripening index (RI), non-protein nitrogen (NPN), electrophoretic and organoleptic properties of the cheese samples were determined at 1, 15, 30 and 60 days of storage. According to the results, the effects of enzyme type on the titratable acidity, dry matter, salt, nitrogenous compounds and all sensory properties, except for the odour was significant ( $p < 0.05$ ). At the end of storage, the titratable acidity, salt, WSN, RI, NPN values and sensory scores of the cheeses increased, while the pH, fat, total nitrogen (TN), protein,  $\beta$ - and  $\alpha_{s1}$ -casein contents of cheeses decreased compared to initial values.

**Key words:** White pickled cheese, calf rennet, recombinant chymosin, microbial rennet.

## INTRODUCTION

Milk-clotting enzymes are the primary active agents in cheesemaking; coagulation of milk is a crucial step, which involves the enzyme-mediated cleavage of  $\kappa$ -casein at the peptide bond Phe 105-Met 106 that renders the casein micelles unstable, and eventually causes aggregation that yields a clot and a gel afterwards (Fox

and McSweeney, 1999; Silva et al., 2003). In addition to such a specific proteolytic activity, milk clotting enzymes usually possess a broader proteolytic activity towards  $\alpha_s$ - and  $\beta$ -caseins, which eventually aids ripening. Commercial calf rennet consists mainly of two proteolytic enzymes, namely chymosin (EC 3.4.23.4) and pepsin.

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**Abbreviations:** WSN, Water soluble nitrogen; NPN, non-protein nitrogen; PPN, proteose-pepton nitrogen; RI, ripening index; EDTA, ethylenediaminetetra acetate; FDM, fat-in-dry matter; TN, total nitrogen.

Chymosin, extracted from abomasa of sucking calves (calf rennet), was the first and still is the most widely used milk-clotting enzyme in traditional cheese-making worldwide (Fox, 1987; Guinee and Wilkinson, 1992; Fox and McSweeney, 1999; Feng et al., 2011). Rennet is used in cheese manufacturing primarily as a milk coagulant. Other enzymes present in rennet also play an important role in cheese production, especially in cheese ripening and may be a cause of the development of bitterness during storage (Rogelj et al., 2001). However, in recent decades, due to a shortage of calf rennet on world markets, alternative milk-clotting enzymes of different origins have been investigated.

The most common rennet substitutes include bovine, porcine and, to a lesser extent, chicken pepsins and microbial proteases from *Mucor miehei*, *Mucor pucillus* and *Cryphonectria parasitica* (formerly *Endothia parasitica*). Proteolytic enzymes of fungal origin have received considerable attention; especially extracellular enzymes of *C. parasitica*, *M. miehei* and *M. pucillus* have received wider acceptability on the industrial scale due to their high milk-clotting and low proteolytic activities (Şeker et al., 1998). Most of the plant coagulants have very high proteolytic capacity and, therefore, they are largely not suitable for cheese-making.

More recently, the gene for chymosin has been cloned and inserted into micro-organisms such as *Escherichia coli* (Chy-Max, Pfizer), *Kluyveromyces lactis* (Maxiren, Gist-Brocades, Delft-Holland) and *Aspergillus niger* var. *awamori* (Chymogen, Genencor) and yeast, resulting in the expression of chymosin for use as a coagulant. The enzymic properties of this biotechnologically derived chymosin are indistinguishable from those of the native calf chymosin, while in cheese manufacture, there are no major differences between cheeses made with the non-animal or calf chymosins (Guinee and Wilkinson, 1992; Fox and McSweeney, 1999; Kosikowski and Mistry, 1997; Broome and Limsowtin, 1998). In addition to the benefit that such chymosin can be produced in large-scale fermentors at low cost, recombinant, highly pure chymosin has also some other advantages such as specific, low proteolytic activity, predictable coagulation behavior, kosher certification, and vegetarian approval (Repelius, 1998; Rogelj et al., 2001).

The aim of this research was to make a comparison between Turkish white brined cheeses manufactured from cow's milk by using alternative coagulating enzyme to commercial and microbial rennets. For this purpose, recombinant chymosin was employed as an alternative coagulant in white pickled cheese production.

## MATERIALS AND METHODS

Cow's milk (morning milking) used in the manufacture of white pickled cheese was collected three times during March 2004 from Holstein cattle in Şanlıurfa. In this study, Rennilase 150 L Type t (from *Rhizomucor miehei* by deep fermentation) and Chy-Max 15 T Plus (from *A. niger* var. *Awamori* by recombinant DNA technology,

100% chymosin) were used as alternative coagulating enzymes. The liquid commercial rennet (90% chymosin+10% pepsin) and alternative coagulating enzymes were supplied by Peyma-Chr. Hansen, Gayrettepe-Istanbul, Turkey. The milks were inoculated with DVS mesophilic homofermentative culture (R-703) consisting of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* (obtained from Peyma-Chr. Hansen, Gayrettepe, Turkey).

## Production of cheese

Cheese was produced at the pilot dairy plant of the Food Engineering Department, Harran University. Two different trials were performed for the manufacture of cheese. In each trial, the large flocks were removed from raw cow's milk using a cloth filter. The milk was heat-treated to 72°C for 2 min, cooled to 32°C, inoculated with starter culture (1% inoculum) and CaCl<sub>2</sub> was added at a rate of 0.02%. After fermentation for 30 min, the milk was divided into three equal portions (30 L each). The first batch (A) was coagulated with liquid commercial rennet (strength, 1: 8000), the second batch (B) was coagulated with Chy-Max (strength, 1: 15000) and third batch (C) was coagulated with Rennilase (strength, 1: 50000). Following coagulation, the coagulum was cut with cutting harps about 1 cm<sup>3</sup> size and drained. The curds were transferred into batches lined with cheese cloth to drain whey and pressed for about 3.5 h at 20±2°C. Then, curd was cut into 7x11x11cm segments to shape and these shaped curds were put into pre-brined solution containing 15% NaCl (w/v) at 18°C for 5 h. Following pre-brining, the cheeses were packaged in plastic cups (1 kg) containing brine (concentration 12%) and transferred to cold storage (4±1°C) for 60 days.

## Analytical methods

The pH of the milk (TSE, 1994) and cheeses (TSE, 1995) was measured using a digital pH-meter (model of Orion 250 A, Orion Research Inc., Boston, USA). The protein content of the milk and cheeses were determined by the Kjeldahl method (Gripon et al., 1975). The total fat and dry matter contents of the cheese samples were determined by the Gerber (TSE, 1994), and gravimetric methods (AOAC, 1990), respectively. Salt content of the cheese was determined by Mohr titration method (Anonymous, 1978) water soluble nitrogen (WSN), non-protein nitrogen (NPN) and proteose-pepton nitrogen (PPN) contents were determined according to Gripon et al. (1975). Ripening index (RI) was estimated by using the formula: (WSN/TN)x 100, as proposed by Alais (1984).

Products of proteolysis in the cheese samples were analyzed by mini urea polyacrylamide gel electrophoresis (Urea-PAGE). Electrophoresis was carried out on a vertical slab unit (Bio-Rad Laboratories, Inc. 1000 Alfred Nobel Drive, Hercules, California, USA) and the stacking gel system described by Creamer (1991). Samples were prepared by grating 0.5 g of each cheese into 25 ml of sample buffer (0.092 g ethylenediaminetetra acetate (EDTA), 1.08 G Tris, 0.55 g boric acid and 36 g urea made up to 100 ml and adjusted to pH 8.4). Each sample was centrifuged at 10000 g for 10 min and 2 ml from the middle portion was taken. All samples were mixed with 3% each of 0.1% (w/v) bromphenol blue solution and mercaptoethanol. 5 µl of the 2% cheese solutions were used for electrophoresis. For dyeing the gels Coomassie Blue R-250 dye solution was used.

The samples were organoleptically assessed by ten panelists using a sensory rating scale of 1-35 for flavour and taste, 1-35 for consistency, 1-20 for odour and 1-20 for color and appearance as described by TSE (1995). The panel of assessors was an external panel of non-smokers who were very familiar with brined cheese

**Table 1.** Physio-chemical properties of Turkish white cheeses produced by using different coagulating enzymes.

Property	Cheese*	Storage period (day)			
		1	15	30	60
pH	A	5.55±0.205 <sup>b3</sup>	5.90±0.042 <sup>b1</sup>	5.92±0.021 <sup>b1</sup>	5.62±0.226 <sup>b2</sup>
	B	5.59±0.057 <sup>ab3</sup>	6.08±0.106 <sup>ab1</sup>	5.97±0.000 <sup>ab1</sup>	5.65±0.007 <sup>ab2</sup>
	C	5.61±0.049 <sup>a3</sup>	5.96±0.042 <sup>a1</sup>	5.99±0.092 <sup>a1</sup>	5.88±0.042 <sup>a2</sup>
Titratable acidity (% lactic acid)	A	0.565±0.021 <sup>a1</sup>	0.465±0.007 <sup>a2</sup>	0.450±0.000 <sup>a2</sup>	0.620±0.014 <sup>a1</sup>
	B	0.515±0.021 <sup>c1</sup>	0.393±0.004 <sup>c2</sup>	0.405±0.007 <sup>c2</sup>	0.540±0.000 <sup>c1</sup>
	C	0.525±0.021 <sup>b1</sup>	0.435±0.035 <sup>b2</sup>	0.425±0.035 <sup>b2</sup>	0.560±0.014 <sup>b1</sup>
Dry matter (%)	A	44.08±0.608 <sup>ab1</sup>	43.24±0.735 <sup>ab2</sup>	42.09±0.665 <sup>ab3</sup>	41.87±0.417 <sup>ab3</sup>
	B	43.37±0.255 <sup>b1</sup>	42.54±0.141 <sup>b2</sup>	41.74±0.191 <sup>b3</sup>	41.88±0.304 <sup>b3</sup>
	C	44.62±0.163 <sup>a1</sup>	43.65±0.184 <sup>a2</sup>	42.54±0.438 <sup>a3</sup>	42.26±0.325 <sup>a3</sup>
Fat (%)	A	21.00±0.000 <sup>a1</sup>	21.25±0.354 <sup>a1</sup>	20.00±0.000 <sup>a2</sup>	20.00±0.000 <sup>a2</sup>
	B	21.00±0.700 <sup>a1</sup>	20.63±0.177 <sup>a1</sup>	20.25±0.354 <sup>a2</sup>	20.25±0.354 <sup>a2</sup>
	C	21.25±1.061 <sup>a1</sup>	21.25±1.061 <sup>a1</sup>	19.75±1.061 <sup>a2</sup>	19.5±0.707 <sup>a2</sup>
Fat in dry matter	A	47.65±0.658 <sup>a1</sup>	49.15±0.021 <sup>b1</sup>	47.52±0.750 <sup>b2</sup>	47.78±0.474 <sup>b2</sup>
	B	48.42±1.351 <sup>b1</sup>	48.49±0.573 <sup>a1</sup>	48.53±1.060 <sup>c2</sup>	48.37±1.195 <sup>c2</sup>
	C	47.63±2.199 <sup>a1</sup>	48.69±2.630 <sup>a1</sup>	46.44±2.970 <sup>a2</sup>	46.16±2.029 <sup>a2</sup>
Protein (%)	A	18.65±0.948 <sup>1</sup>	17.21±0.912 <sup>2</sup>	16.95±0.884 <sup>2</sup>	16.55±0.679 <sup>2</sup>
	B	17.99±0.410 <sup>1</sup>	17.08±0.120 <sup>2</sup>	16.65±0.389 <sup>2</sup>	16.47±0.594 <sup>2</sup>
	C	18.86±0.940 <sup>1</sup>	17.46±1.365 <sup>2</sup>	17.59±1.407 <sup>2</sup>	17.29±1.110 <sup>2</sup>
Salt (%)	A	3.13±0.078 <sup>b3</sup>	3.61±0.240 <sup>b2</sup>	3.84±0.318 <sup>b2</sup>	4.14±0.205 <sup>b1</sup>
	B	3.46±0.014 <sup>a3</sup>	3.86±0.085 <sup>a2</sup>	3.99±0.042 <sup>a2</sup>	4.26±0.042 <sup>a1</sup>
	C	3.33±0.127 <sup>a3</sup>	3.94±0.042 <sup>a2</sup>	4.07±0.035 <sup>a2</sup>	4.32±0.106 <sup>a1</sup>

\*A, Manufactured by using commercial liquid rennet; B, manufactured by using recombinant chymosin (*A. niger* var. *awamori*); C, manufactured by using Rennilase (*R. miehe*); \*\*Different letters in the same column indicate significant differences among the samples depending on enzyme type, and different numbers in the same line indicate significant differences among the samples depending on storage time ( $p < 0.05$ ).

and were checked on the basis of sensory acuity and consistency. The experiment was designed according to a 3x4 factorial design. The data were analysed statistically by means of SPSS statistical software program (version 5.0). Statistically different groups were determined by the least significant difference (LSD) test (Bek and Efe, 1995).

## RESULTS AND DISCUSSION

### Physico-chemical characteristics

The chemical composition of the cow's milk used to produce the cheese (data not shown) fell within the following averages ( $n=2$ ): titratable acidity 0.17 ( $\pm 0.013$ )% lactic acid, pH 6.64 ( $\pm 0.071$ ), dry matter 12.59 ( $\pm 0.156$ )%, fat 3.20 ( $\pm 0.141$ )%, protein 3.91 ( $\pm 0.106$ )% and lactose 4.73 ( $\pm 0.04$ )%. Variation in physio-chemical properties of the white cheeses throughout ripening period are shown

in Table 1.

The data reveals that the coagulating enzyme type had significant effect on titratable acidity, dry matter and salt content in cheese throughout the ripening time at 4°C. At the beginning of storage, the titratable acidity value in the cheese A was the highest. Titratable acidity of samples decreased during storage up to day 30, and then increased; this trend could be associated with diffusion of lactic acid from the cheese into the brine. Similar results were reported by Saldamlı and Kaytanlı (1998) and Irigoyen et al. (2001).

The pH values of the ripened cheeses ranged between 5.62 to 5.88. The trends in the variation of pH values over the ripening period were similar for all batches. In all cheese batches, the pH increased until day 30 of ripening, after which it decreased slightly. Similar finding were reported earlier (Irigoyen et al., 2001) and could be attributed to the breakdown of lactic acid when all the

**Table 2.** Nitrogen fractions of Turkish white cheeses produced by using different coagulating enzymes (%).

Property	Cheese*	Storage period (day)			
		1	15	30	60
Total nitrogen	A	2.923±0.148 <sup>1</sup>	2.696±0.143 <sup>2</sup>	2.656±0.139 <sup>2</sup>	2.594±0.107 <sup>2</sup>
	B	2.820±0.064 <sup>1</sup>	2.677±0.019 <sup>2</sup>	2.609±0.061 <sup>2</sup>	2.582±0.093 <sup>2</sup>
	C	2.956±0.148 <sup>1</sup>	2.736±0.214 <sup>2</sup>	2.756±0.221 <sup>2</sup>	2.709±0.174 <sup>2</sup>
Water soluble nitrogen (%)	A	0.325±0.016 <sup>b1</sup>	0.337±0.015 <sup>b2</sup>	0.345±0.016 <sup>b3</sup>	0.374±0.020 <sup>b4</sup>
	B	0.308±0.014 <sup>c1</sup>	0.311±0.016 <sup>c2</sup>	0.323±0.015 <sup>c3</sup>	0.360±0.014 <sup>c4</sup>
	C	0.348±0.017 <sup>a1</sup>	0.363±0.022 <sup>a2</sup>	0.373±0.024 <sup>a3</sup>	0.411±0.032 <sup>a4</sup>
Ripening index (%)	A	11.11±0.007 <sup>b1</sup>	12.49±0.106 <sup>b2</sup>	12.98±0.064 <sup>b3</sup>	14.42±0.170 <sup>b4</sup>
	B	10.92±0.255 <sup>c1</sup>	11.62±0.495 <sup>c2</sup>	12.36±0.283 <sup>c3</sup>	13.95±0.049 <sup>c4</sup>
	C	11.78±0.021 <sup>a1</sup>	13.26±0.233 <sup>a2</sup>	13.54±0.212 <sup>a3</sup>	15.15±0.205 <sup>a4</sup>
Non-protein nitrogen	A	0.163±0.008 <sup>b1</sup>	0.171±0.004 <sup>b2</sup>	0.211±0.013 <sup>b3</sup>	0.234±0.019 <sup>b4</sup>
	B	0.144±0.007 <sup>c1</sup>	0.151±0.006 <sup>c2</sup>	0.180±0.004 <sup>c3</sup>	0.189±0.008 <sup>c4</sup>
	C	0.182±0.005 <sup>a1</sup>	0.192±0.011 <sup>a2</sup>	0.223±0.009 <sup>a3</sup>	0.237±0.007 <sup>a4</sup>

\*A, Manufactured by using commercial liquid rennet; B, manufactured by using recombinant chymosin (*A. niger* var. *awamori*); C, manufactured by using Rennilase (*R. miehei*); Different letters in the same column indicate significant differences among the samples depending on enzyme type, and different numbers in the same line indicate significant differences among the samples depending on storage time ( $p < 0.05$ ).

residual lactose has been metabolized and the production of basic substances is completed (McSweeney and Fox, 1993), and to the metabolization of the non-protein nitrogen components and organic acids (Farkye and Fox, 1990). Salt content of the cheese A was lower than the other cheeses. Salt contents in the experimental cheeses increased during the ripening period because of diffusion of salt from the surfaces into the centre of the cheese where samples were taken for these determinations.

Total protein content of the cheeses decreased slightly over the ripening period, with values being comparable to the results previously reported for white pickled cheese (Saldamlı and Kaytanlı, 1998). The fat content remained steady in all cheese batches throughout the ripening period. In addition, the moisture and fat-in-dry matter (FDM) contents of all ripened cheeses met the figures given by the Turkish Standardization Institute (TSE) for the first quality white pickled cheese (TSE, 1995). Dry matter contents in the cheese B were lower than cheeses A and C. It could be deduced that the recombinant chymosin made a structure with the milk components that retained more liquid during the wheying stage of the cheese. Dry matter contents in white cheeses decreased during the ripening period because of new occurring ionic group as a result of breaking the peptide bond (Creamer and Olson, 1982) and also increasing water binding capacity of protein in cheese which is stored at low temperature (Salama et al., 1982). These results are in agreement with those reported by Saldamlı and Kaytanlı (1998) and indicated that the cheeses A, B and C did not

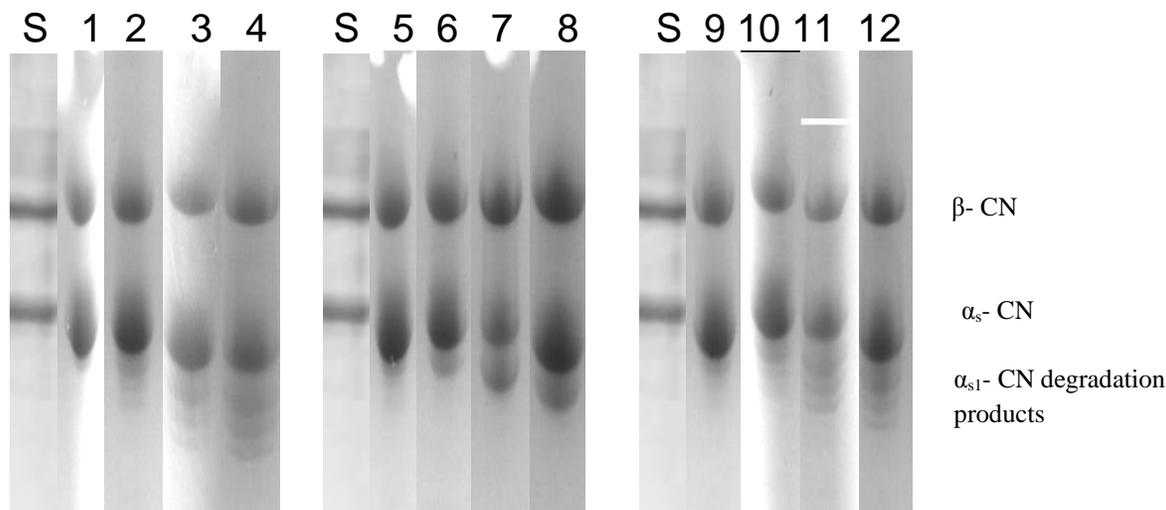
significantly differ ( $p > 0.05$ ) in fat, fat-in-dry matter and protein content throughout ripening. Also, the coagulating enzymes did not affect pH, protein, fat or fat in dry matter contents of white cheeses significantly ( $p > 0.05$ ).

### Nitrogen fractions

The WSN values increased throughout ripening in all cheese samples. The different milk-clotting enzymes employed yielded significant differences in the WSN values (Table 2); the cheese made with the *R. miehei* (cheese C) had higher WSN values. The WSN values were similar to the values recorded for the same cheese variety by Saldamlı and Kaytanlı (1998). Ripening index (WSN/total nitrogen (TN)) values (ripening index) increased throughout ripening in all cheese samples, however, RI levels were significantly higher ( $p < 0.05$ ) in the cheese C. The formation of water soluble nitrogen compounds during ripening is an indicator of casein hydrolysis brought about by the action of the rennet and the milk proteases present at the start of ripening (Irigoyen et al., 2001). Therefore, WSN value is an index of the rate and extent of proteolysis. The NPN values increased continuously over the ripening period in all cheeses (Table 2). The cheeses made by using *A. niger* var. *awamori* (cheese B) showed lower NPN values.

### Electrophoresis of the caseins

The  $\alpha$ - and  $\beta$ -casein underwent a significant decrease



**Figure 1.** Urea-PAGE electrophoretograms of bovine sodium caseinate and white pickled cheese manufactured using commercial calf rennet (lanes 1-4), recombinant chymosin (lanes 5-8) and microbial rennet (lanes 9-12) after 1, 15, 30 and 60 day of ripening.

during ripening (Figure 1). This was a result of the action of the residual rennet in combination with the action of the hydrolytic enzymes of the microorganisms present in the cheeses (Irigoyen et al., 2001). The first casein fraction to be broken down was the  $\alpha_{s1}$ -casein and  $\alpha_{s1}$ -casein degradation products in all cheese samples at different rates. The influence of the milk-clotting enzyme on the  $\alpha_s$ -casein was more pronounced. This finding was in agreement with the results reported by Saldamlı and Kaytanlı (1998), who employed capillary electrophoresis to study the differential degradation of  $\alpha_s$ -casein by various coagulants. At the end of the ripening period, the bands representing residual  $\alpha_{s1}$ -casein and  $\alpha_{s1}$ -casein degradation products in the cheese made using *A. niger* var. *awamori* (cheese B) were less visible than that of the cheeses A and C. The  $\alpha_{s1}$ -casein fraction is the fraction most intensely broken down by rennet proteases during ripening (Farkye and Fox, 1990).

Leu190-Tyr191 and Ala187-Phe188 are the regions on the  $\beta$ -casein that are most susceptible to the action of chymosin (Whyte, 1995). Breakdown of the  $\beta$ -caseins was less pronounced than that of the  $\alpha_s$ -caseins in both the experimental cheese batches. Several other workers have previously reported greater resistance of  $\beta$ -caseins to enzymatic hydrolysis (Fox and Law, 1991). In this study, the degree of  $\beta$ -casein degradation differed between the coagulating enzymes tested. At the end of the ripening period, the residual  $\beta$ -casein in the cheese B was more visible than the cheeses A and B.

### Sensory analysis

Table 3 shows the sensory attributes evaluated by the

panel group for each cheese samples. The cheese B (made by using *A. niger* var. *awamori*) received highest scores for colour and appearance, odour and flavour at the end of storage. Total sensory scores (colour and appearance, body and texture, odour and flavour) of the cheese B were close to the cheese C and both had higher sensory scores than the cheese A. During storage, total sensory scores of all the cheeses increased. The results of the sensory analysis plainly showed the appreciable influence of ripening time, with the cheeses becoming more moist and tender with higher adherence and characteristic texture scores after 60 days.

### Conclusions

Coagulating enzyme types and storage period had significant effects on the titratable acidity, dry matter and the sensory characteristics of the cheeses. The titratable acidity and dry matter content of the cheeses made by using *A. niger* var. *awamori* (cheese B) were lower than the other cheeses and they received the highest sensory scores from the panelists.

The bands corresponding to nitrogen fractions were less visible in the cheese B; and proteolytic activity on the  $\beta$  and  $\alpha_{s1}$ -caseins, especially on the  $\alpha_{s1}$ -caseins, was likewise lower. Thus, both the formation and the subsequent degradation of the  $\alpha_{s1}$ -casein took place less rapidly in the cheeses made by using *A. niger* var. *awamori* (cheese B). According to the results obtained, it could be speculated that the overall quality of the cheese made with *A. niger* var. *awamori* (cheese B) was quite similar to the the cheeses made with commercial calf rennet or microbial rennet. The cheese B had the lowest

**Table 3.** Sensory characteristics of Turkish white cheeses produced by using different coagulating enzymes (%).

Property	Cheese*	Storage period (day)			
		1	15	30	60
Color and appearance	A	19.28±0.014 <sup>b1</sup>	18.92±0.007 <sup>b12</sup>	19.06±0.276 <sup>b2</sup>	19.14±0.191 <sup>b12</sup>
	B	18.91±0.134 <sup>a1</sup>	19.79±0.297 <sup>a12</sup>	19.32±0.332 <sup>a2</sup>	19.53±0.106 <sup>a12</sup>
	C	20.35±0.205 <sup>a1</sup>	19.27±0.262 <sup>a12</sup>	18.97±0.191 <sup>a2</sup>	19.28±0.113 <sup>a12</sup>
Body and texture	A	28.45±0.318 <sup>b2</sup>	32.81±0.205 <sup>b1</sup>	32.90±0.255 <sup>b1</sup>	32.90±0.141 <sup>b1</sup>
	B	30.47±0.757 <sup>b2</sup>	33.47±0.156 <sup>b1</sup>	31.64±0.396 <sup>b1</sup>	32.43±0.099 <sup>b1</sup>
	C	33.58±0.339 <sup>a2</sup>	31.69±0.339 <sup>a1</sup>	33.66±0.219 <sup>a1</sup>	33.28±0.247 <sup>a1</sup>
Odour	A	9.28±0.361 <sup>2</sup>	9.70±0.170 <sup>1</sup>	9.52±0.092 <sup>12</sup>	8.67±0.042 <sup>3</sup>
	B	9.27±0.000 <sup>2</sup>	9.69±0.198 <sup>1</sup>	9.44±0.021 <sup>12</sup>	8.80±0.283 <sup>3</sup>
	C	9.470±0.283 <sup>2</sup>	9.53±0.035 <sup>1</sup>	9.435±0.021 <sup>12</sup>	8.68±0.318 <sup>3</sup>
Flavour	A	29.28±0.071 <sup>b3</sup>	29.26±0.014 <sup>b3</sup>	30.78±0.078 <sup>b1</sup>	31.25±0.219 <sup>b2</sup>
	B	31.61±0.198 <sup>a3</sup>	31.64±0.269 <sup>a3</sup>	32.36±0.396 <sup>a1</sup>	31.28±0.389 <sup>a2</sup>
	C	28.77±0.438 <sup>b3</sup>	29.28±0.389 <sup>b3</sup>	31.39±0.042 <sup>b1</sup>	30.40±0.566 <sup>b2</sup>
Total score	A	86.28±0.042 <sup>b3</sup>	90.66±0.354 <sup>b2</sup>	92.25±0.700 <sup>b1</sup>	91.95±0.071 <sup>b2</sup>
	B	90.25±1.089 <sup>a3</sup>	94.59±0.325 <sup>a2</sup>	92.75±0.354 <sup>a1</sup>	92.03±0.467 <sup>a2</sup>
	C	92.71±0.071 <sup>a3</sup>	89.76±0.346 <sup>a2</sup>	93.45±0.389 <sup>a1</sup>	91.63±0.523 <sup>a2</sup>

\*A, Manufactured by using commercial liquid rennet; B, manufactured by using recombinant chymosin (*A. niger* var. *awamori*); C, manufactured by using Rennilase (*R. miehei*). Different letters in the same column indicate significant differences among the samples depending on enzyme type, and different numbers in the same line indicate significant differences among the samples depending on storage time (p<0.05).

level of proteolysis and received higher sensory scores than the other cheeses during storage. Consequently, it is thought that recombinant chymosin can be successfully used in white pickled cheese production as an alternative coagulant to the commercial calf rennet and microbial rennet. Therefore, this would allow the cheese consumption by consumers who object to use of animal-derived products in cheese for religious, moral and dietary reasons.

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Full Length Research Paper

## Development of polyclonal antibodies for the detection of recombinant human erythropoietin

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**Recombinant human erythropoietin (rHuEPO) is detected by using direct pharmacological assays and indirect haematological assays. However, both methods have several limitations including technical challenges and cost-related issues. The aim of this study was to develop polyclonal antibodies against rHuEPO (anti-rHuEPO pAb) that can be used in immunoassays. In this study, we purified anti-rHuEPO pAb that could be used in immunoblotting assays to efficiently detect rHuEPO. Furthermore, these anti-rHuEPO pAb which could also detect rHuEPO that was expressed in a eukaryotic expression system (CHO cells). Thus, the anti-rHuEPO pAb developed in this study may be useful for rHuEPO detection.**

**Key words:** Antibodies, rHuEPO, immunoassays, pAb.

### INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that is responsible for the homeostatic regulation of red blood cell production, and consequently increases tissue oxygenation (Lacombe and Mayeux, 1998). Human recombinant erythropoietin (rHuEPO) has been successfully produced in mammalian cells cultures since the late 1980s and has several therapeutic applications such as the treatment of anaemia and polycythemia in patients

having chronic kidney disease, acquired immunodeficiency syndrome (AIDS) and cancer (Macdougall and Ashenden, 2009). rHuEPO was used as a performance enhancement drug by athletes participating in endurance sports, has therefore been banned by the World Anti-Doping Agency since 1990 (Reichel and Gmeiner, 2010). Thus, rHuEPO detection methods are used for evaluating blood doping and for disease

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diagnosis. The increased availability of biosimilars and uncontrolled drugs has resulted in a need for the development of reliable rHuEPO detection methods (Girard et al., 2012).

The rHuEPO detection assays either use a direct pharmacological approach, or an indirect haematological approach. However, both methods have several limitations (Diamanti-Kandarakis et al., 2005) including technical challenges and cost-related issues (Azzazy et al., 2005). Recently, several rHuEPO assays were developed for studying the pathophysiology of anaemia and polycythemia (Lonnberg et al., 2012). Immunoassays that use antibodies against rHuEPO have been immensely useful for studying the structure/function of EPO and for the sensitive detection of EPO in biological fluids (Bornemann et al., 2003; Mi et al., 2005; Sytkowski and Fisher, 1985; Wang et al., 2003).

Cell lines such as the Chinese hamster ovary (CHO) cell line are commonly used for producing recombinant human glycoproteins (Ghaderi et al., 2012). CHO cells have become a popular alternative to animals as an expression system for the efficient production of high quality recombinant proteins. Moreover, the glycosylation machinery of CHO cells is similar to that of human cells (Jeong et al., 2008; Stanley et al., 1996). The objective of this study was to develop polyclonal antibodies for rHuEPO detection.

## MATERIALS AND METHODS

Two 6-month-old male New Zealand rabbits were immunized using rHuEPO following a 30-day adaptation period. Five subcutaneous injections were administered in the scapular area of each rabbit, alternating between the right and left sides. The first immunization dose contained 84 µg rHuEPO and complete Freund's adjuvant (Sigma-Aldrich, USA). Subsequent immunizations were performed after 7, 14, 21 and 28 days using rHuEPO (84 µg) and incomplete Freund's adjuvant (Sigma-Aldrich, USA). Prior to immunization, blood was collected to determine the antibody titres. After the last immunization, indirect enzyme linked immuno sorbent assay (ELISA) was used to determine the rHuEPO antibody titres. Hyperimmune sera were obtained from animals with high antibody titre. The hyperimmune serum was stored at -20°C until required for further processing and purification. The antibodies were purified by affinity chromatography using a protein A-Sepharose CL-4B column (GE Healthcare Company, USA) according to the manufacturer's instructions. The animals used in this study were treated in accordance with the guidelines recommended by Colégio Brasileiro de Experimentação Animal. The rHuEPO used in all experiments was EPREX<sup>®</sup> by Janssen Cilag (Issy-les-Moulineaux, France).

For performing the rHuEPO ELISA, polystyrene ELISA microtitre plates (NuncMaxiSorp<sup>®</sup>, NalgeNunc International, USA) were coated with rHuEPO (50 ng/well) and incubated overnight at 4°C. Next, the plates were washed with phosphate buffer saline with 0.05% Tween 20 (PBS-T). Then, the wells were treated with blocking buffer (PBS containing 5% skim milk). Serial dilutions of the purified anti-rHuEPO pAb were incubated with rHuEPO-coated wells. To detect the rHuEPO pAb complex, goat anti-rabbit antibody

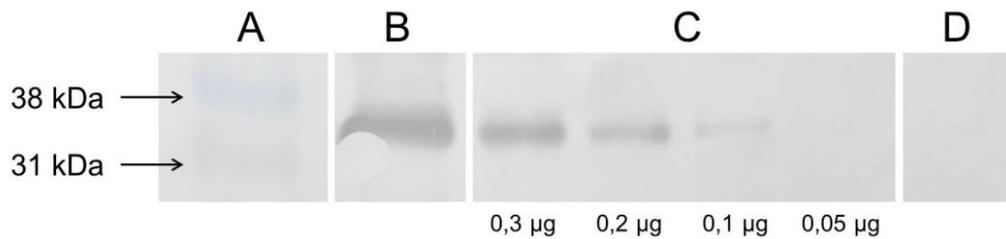
labelled with Ig-peroxidase conjugate (Sigma-Aldrich, USA) was added to the well. A substrate solution containing *o*-phenylenediamine (0.4 mg/mL in 0.1 M citrate buffer, pH 5.0) and 0.03% H<sub>2</sub>O<sub>2</sub> was added to the ELISA plate and incubated for 15 min. The reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub> (3 N), and the optical densities of the solutions were measured at 492 nm using the VICTOR<sup>™</sup> X5 Multilabel Plate Reader (Perkin Elmer, USA).

Immunoblotting was performed to evaluate the specificity and sensitivity of the polyclonal antibodies. Rabbit preimmune sera and anti-EPO rabbit antibody (Sigma-Aldrich, USA) were used as the negative and the positive controls, respectively. rHuEPO solutions with concentrations of 0.05-0.3 µg per well were loaded onto sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) gels. For positive and negative controls, rHuEPO concentration of 1 µg per well was used. After electrophoresis, the rHuEPO proteins were transferred onto a nitrocellulose membrane (Millipore, USA) and then incubated at 37°C for 1 h with anti-rHuEPO pAb that was diluted 1:10,000. Next, goat anti-rabbit Ig-peroxidase conjugate (Sigma-Aldrich, USA) was added. The bands were visualized by using substrate/chromogen solution (0.6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl at pH 8.0, and 0.03% H<sub>2</sub>O<sub>2</sub>).

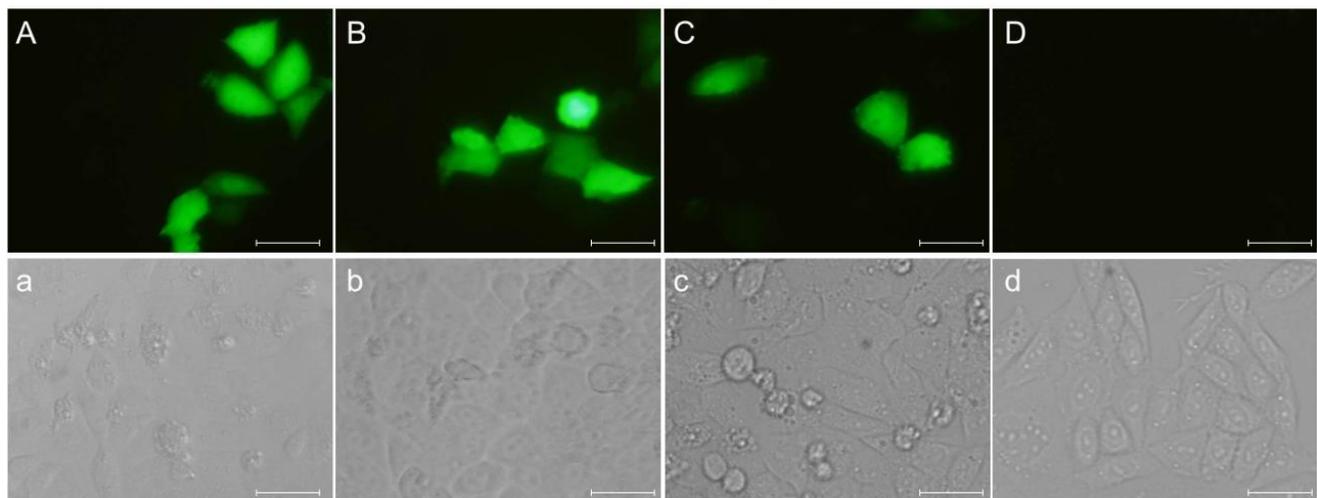
The Chinese hamster ovary (CHO)-K1 cell line was purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and grown at 37°C in 5% CO<sub>2</sub>. Prior to transfection, the cells were seeded in 96-well plates (10<sup>4</sup> cells/well) and incubated until 80% confluence was reached. pTarget/EPO plasmid construct (Collares et al., 2012), pTARGET<sup>™</sup> (Promega, USA) and pEGFP (Clontech, USA) were prepared for transfection according to the manufacturer's instructions (Lipofectamine<sup>®</sup> 2000 Transfection Reagent - Life Technologies, USA). The cells were washed twice with PBS and incubated for 4 h with lipoplex mixture. After 24 h, the lipoplexes were removed by aspiration and the cells were fixed using methanol. The cells were then washed twice with PBS, blocked by using PBS with 10% fetal bovine serum and then incubated with anti-rHuEPO pAb for 2 h. After washing, the cells were incubated with goat anti-rabbit FITC conjugated antibodies (Invitrogen, USA) for 1 h. The fluorescent labels were visualized by using a fluorescence microscope (Olympus BX 71) with an excitation wavelength of 450 nm. CHO cells transfected with pEGFP and stained with a commercially available anti-EPO antibody (Sigma-Aldrich, USA) were used as positive controls; CHO cells transfected with pTARGET and stained with rabbit sera collected before immunization were used as negative controls.

## RESULTS AND DISCUSSION

Several manufacturers produce anti-rHuEPO. However, commercially available anti-rHuEPO antibodies remain expensive, and the appearance of new competing manufacturers still has not led to price reduction and solution doping. Considering the high cost for production of monoclonal antibodies, the objective of this study was to produce cost-effective anti-rHuEPO pAb that can be used for immunoblotting and immunofluorescence assays. First, the anti-rHuEPO pAb was purified and used to perform indirect ELISA. The ELISA data indicated that rHuEPO could be efficiently detected using high antibody dilutions (1:10,000). To evaluate the anti-rHuEPO



**Figure 1.** rHuEPO detection by anti-recombinant human erythropoietin polyclonal antibody (rHuEPO pAb) in an immunoblotting assay. **A**, Molecular weight standard; **B**, positive control; **C**, anti-rHuEPO pAb (1:10,000); **D**, negative control.



**Figure 2.** Immunofluorescence analysis of anti-rHuEPO pAb in CHO cells transfected with pTARGET/EPO. Panels: **A**, Transfection control (pEGFP); **B**, specific staining of CHO with commercial anti-EPO antibody; **C**, specific staining of CHO with anti-rHuEPO pAb diluted 1:10,000; **D**, antibody negative control. Phase contrast (a, b, c and d) microphotographs was used. Scale bars represent 100 µm.

pAb detection sensitivity, an immunoblotting assay was performed using various concentrations of rHuEPO. The anti-rHuEPO pAb (1:10,000) detection limit was determined to be 0.1 µg rHuEPO. Bands corresponding to the molecular weight of rHuEPO (30 kDa) were visualized (Figure 1C). Similar results were obtained with the commercial anti-EPO antibody (Figure 1B). In contrast, the rabbit sera obtained before immunization failed to detect rHuEPO (Figure 1D). Since the erythropoietin forms epoetin  $\alpha$  and epoetin  $\beta$  are both produced in CHO cell lines and the basis for detecting rHuEPO doping relies on the glycosylation pattern of the protein (Franz, 2009; Girard et al., 2012; Reichel and Gmeiner, 2010), we hypothesized that anti-rHuEPO pAb could detect rHuEPO obtained from a eukaryotic expression system. To evaluate this hypothesis,

pTARGET/EPO transfected CHO cells were stained with either anti-rHuEPO pAb (Figure 2C) or the commercial anti-EPO antibody (Figure 2B) to detect rHuEPO expression. The preimmunization sera failed to detect rHuEPO in pTARGET/EPO-transfected CHO cells (Figure 2D) and on pTARGET transfected CHO cells (data not shown). To evaluate the transfection efficiency and rHuEPO expression capability of CHO cells, pEGFP was used as a transfection control (Figure 2A).

The anti-rHuEPO pAb specifically detected rHuEPO by immunofluorescence staining and immunoblotting, which are methods that are commonly used to measure protein expression levels and to evaluate physiological roles of proteins (Ben-Gedalya et al., 2011; Brown et al., 1998; Valdenaire et al., 1999). Polyclonal antibody production offers a rapid and cost-effective alternative to commer-

cially available monoclonal antibodies. In addition, polyclonal antibodies are known to recognize several epitopes on the same antigen and therefore detect antigens more efficiently than monoclonal antibodies. In conclusion, the anti-rHuEPO pAb produced in this study may be useful for various rHuEPO detection assays.

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*Full Length Research Paper*

## Alteration in lipid metabolism induced by a diet rich in soya-oil and amylopectin in a rat model

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This present study was designed to evaluate the impact of different dietary regimens on lipid metabolism in brain, liver and plasma of albino rat model. Twenty (20) male Wister albino rats (110 g) were assigned to two dietary groups and housed individually. One group received a control diet enriched in 20% soy- oil and another enriched with 20% amylopectin. The starved group was fed primarily with carboxyl methyl cellulose (CMC). Daily growth rate and average condition factor of each group was monitored for 14 days. Lipid profiles of brain, liver and plasma were analyzed by colorimetric, immunological, thin layer chromatography and gas chromatography-mass spectrometry. Elevated serum total cholesterol was indicated in rats of starved, oil fed and starch fed rats by factors of 0.40, 0.65 and 0.75 mmol/L respectively when compared with the control. Low density lipoprotein (LDL) cholesterol was 0.9, 1.1 and 0.95 mmol/L higher in starved, oil fed and starch fed rat groups, respectively when compared with the control group. Alteration in feeding pattern revealed a marked decrease in the levels of the steroidal sex hormones in the starved and other dietary groups compared to the control. Hepatic and liver lipid profile revealed the polar lipids tentatively identified altered expression of diphosphatidyl glycerol, phosphatidyl choline, phosphatidyl serine, and cholesterol in order of decreasing polarity. GC-MS results showed a shift in the ratio of saturated to unsaturated fatty acids in the test groups. Lauric acid was observed to be predominantly present in the starved group.

**Key words:** Lipid metabolism, soy-oil, steroidal hormones, liver lipid, serum lipoproteins.

### INTRODUCTION

Dietary intake has a lot of influence on our nutrition and health. The incidence of cardiovascular disease (CVD) and diabetes type 2 is increasing worldwide representing the two most influential lifestyle-dependent diseases at present (Anna et al., 2012). In addition, lipid droplets are implicated in a number of other cellular functions, ranging from protein storage and degradation to viral replication. These processes are functionally linked to many physiological and pathological conditions, including obesity and

related metabolic diseases (Tobias and Robert, 2012). Consequently, multiple components of these metabolic syndrome including obesity and diabetes have been linked with Alzheimers disease (Frisardi et al., 2010; Luchsinger et al., 2012; Whitmer et al., 2008) and considerable evidence also implicate them in cognitive decline (Biessels et al., 2008; Talbot et al., 2012). Different feeding pattern is usually accompanied by pathophysiological conditions in affected organism and

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**Abbreviations:** CVD, Cardiovascular disease; TGs, triglycerides; CNS, central nervous system; CMC, carboxyl methyl cellulose; HDL-C, high density lipoprotein C; LDL-C, low density lipoprotein C; EDTA, ethylenediaminetetra acetate; ELISA, enzyme linked immuno sorbent assay; DPG, diphosphatidyl glycerol; PC, phosphatidylcholine; PS, phosphatidyl serine; CL, cholesterol; NL, neutral lipids; GC/MS, gas chromatography - mass spectra; ADP, adenosine di phosphate; ATP, adenosine tri phosphate.

consequent serious morbidity. A high dietary fiber intake has been proposed to reduce the risk of diabetes type 2 ((Weickert and Pfeiffer, 2008) through improvement of glycemic and insulinemic response and also reduced insulin resistance (Ulmius et al., 2009).

The hydrophobic core of neutral lipids stores metabolic energy. Fatty acids liberated from triglycerides (TGs) in lipids have one of several fates. They can be re-esterified to TG, used for  $\beta$ -oxidation to generate energy, used as building blocks for membrane lipid synthesis, or used as cofactors for cell signaling or exported (Tobias and Robert, 2012). Lipid metabolism is of particular interest due to its high concentration in the central nervous system (CNS). The importance of lipids in cell signaling and tissue physiology is demonstrated by many CNS disorders and injuries that involve deregulated metabolism (Rao and Hatcher, 2007).

These resultant alterations in lipid metabolism might lead to multiple deleterious changes in cellular function that are prominent features of many lipid-related diseases in the societies. To critically assess the role of alterations in lipid metabolism and the role of the accumulation of lipids in organ, it would be necessary to identify the types of lipid molecular species that accumulate and next correlate their presence and abundance to pathological changes in cellular function. This will facilitate early diagnosis of disease and potentially provide a valuable measure of treatment efficacy.

Although considerable efforts have been directed at evaluating alterations in various metabolic and physiological functions, lipid metabolism in response to different feeding pattern in the rat -by extension human- has not been fully evaluated.

Previous lipid analytical approaches have been limited and lack a detailed analysis of specific lipid classes and their associated fatty acid metabolites. It is expedient to know the changes taking place in the various lipid composition of an organism as this plays a major role in the adaptation of animal to stress and in their respective cognitive performance.

In this study, we began with the identification and quantitation of different lipid classes in individual molecular species to determine the biochemical flux through cellular metabolic networks. We also analyzed and compared the lipid changes induced by different feeding patterns and starvation using thin layer chromatography and gas chromatography-mass spectrometry. A connection between the lipid profile changes with meaningful pathological conditions of adaptation to stress, and cognitive function was observed in albino rats.

## MATERIALS AND METHODS

### Chemicals

Diphosphatidyl glycerol, phosphatidyl choline, phosphatidyl serine, di-isobutyl ketone, ethanol, acetic acid, ethylacetate, cyclohexane, chloroform, methanol, and isopropanol were purchased from Sigma

Chemical Co. (St. Louis, MO), USA. Precoated silica gel plates type F254 from Merck A. G. (Darmstadt, Germany). Bovine serum albumin (BSA) was R.I.A grade (Fraction V), pure steroids standard (11- ketotestosterone, testosterone, and estradiol) and the secondary antibody (Mouse monoclonal anti rabbit IgG) was purchased from SpiBio (Saclay, France). Ammonia solution was obtained from Aldrich (Steinheim, Germany). Triton X-100 was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). All other organic solvents were of analytical grade.

### Animals and diets

Male Wistar rats of average weight 110 g were used according to the standard guideline of the Committee on care and use of experimental animal resources. They were housed one per cage, in wire-bottomed cages in a temperature-controlled room (22°C) with a 12-h light: dark cycle and maintained on a standard diet with water *ad libitum* for six weeks

### Feeding pattern

Rats were randomly divided into four groups of five rats per group and fed for two weeks. The composition of this diet is shown in Table 1. Diet formulation was done as earlier described (Zulet et al., 1999). Rats (n=5) in groups A, B, C and D were fed with control, soybean oil enriched, starch rich and carboxyl methyl cellulose (CMC) diets, respectively. The weight of the rats in each group was monitored daily for 14 days. Mathematical model of Chatelier et al. (2006) was used for the evaluation of daily growth and condition factor as shown below:

$$\text{Daily growth} = \{((M_f - M_i)/M_i) \times 100\} / n$$

$$\text{Condition factor} = (M_f/BL^3) \times 100$$

Where,  $M_f$  is final mass in kg,  $M_i$  is initial mass in kg,  $n$  is the number of feeding days and  $BL$  is body length in cm.

At the end of the experiment, the animals were sacrificed by decapitation. Blood was quickly collected and organs (brain and liver) isolated. Serum was separated from blood samples and was used immediately. The brain and liver from all rats were removed, weighed and frozen at -10°C until use.

### Serum lipid profile

The blood serum obtained from the animals was analyzed for total cholesterol, triglycerides, high density lipoprotein C (HDL-C), and low density lipoprotein C (LDL-C). Chemical assays were performed by utilizing commercially available kit (Randox UK). Instrument set-up, run procedures, and maintenance policies were strictly adhered according to the manufacturer's instructions. Total cholesterol was measured after release from its esters by an ester hydrolase using the cholesterol oxidase technique. Triglyceride level was measured after hydrolysis by lipoprotein lipase by assay of released glycerol. LDL-C was calculated according to the method of Friedewald (1972).

### Steroid sex hormones assay

Immunological measurement of sex steroids was carried out using 0.5 mL aliquots of plasma. Plasma was thawed and the free steroids were extracted twice with 2 ml of a mixture made of ethylacetate/cyclohexane (1:1). After freezing at -4°C, the organic

**Table 1.** Feed formulation

Feed composition	Control ( Group A)	Group B	Group C	Group D
Corn Starch (g/100 g)	42.2	42.2	42.2	-
Skimmed milk (g/100 g)	43.8	43.8	43.8	-
Premix (g/100 g)	4	4	4	-
Pure Starch ( g/100 g)	-	10	-	-
Soy oil (g/100 g)	-	-	10	-
CMC ( g/100 g)	-	-	-	100

1 g of premix contains: 3200 i.u vitamin A, 600 i.u vitamin D3, 2.8 mg vitamin E, 0.6 mg vitamin K3, 0.8 mg vitamin B6, 0.004 mg vitamin B12, 0.2 mg folic acid, 0.1 mg biotin H2, 70 mg choline chloride, 0.08 mg Cobalt, 1.2 mg Copper, 0.4 mg iodine, 8.4 mg iron, 16 mg manganese, 0.08 mg selenium, 12.4 mg zinc, 0.5 mg antioxidant. Skimmed milk contains 28% protein.

phase was collected in 5 ml glass tubes and dry evaporated using a Speed-Vacuum apparatus. The dry residue was then dissolved in 0.5 ml of assay buffer (potassium phosphate buffer 0.1 M, pH 7.4, 0.9% (w:v) NaCl, 1 mM ethylenediaminetetra acetate (EDTA), 0.1% (w:v) bovine serum albumin (BSA) and 0.01% NaN<sub>3</sub>). Each sample was analyzed in triplicate (Maclouf et al., 1987). Standard curve was obtained using pure 11KT dissolved in assay buffer, and ranging from 7.8 to 1000 pg/ mL. The crossing activity of specific anti-11KT antibody, (rabbit anti- 11KT serum) was as described in the instruction manual (Maclouf et al., 1987). Enzyme linked immuno sorbent assay (ELISA) for estradiol were adapted from schedule previously described for 11-ketotestosterone (Nash et al., 2000).

#### Thin layer chromatography

Lipids were extracted as described elsewhere (Bligh and Dyer, 1959). The inclusion of isopropanol rather than methanol in the extraction avoids any lipid degradation by endogenous lipolysis. Analytical thin layer chromatography (TLC) was carried out using silica gel plates (E. Merck, Darmstadt, Germany). One dimensional polar lipid separations were achieved using a solvent system of Diisobutylketone: chloroform: methanol: acetic acid: distilled water (3: 4.5: 1.5: 2: 0.4 by vol.) Lipids were visualized by brief exposure in an iodine tank, or using a spray of 8-anilino-1- naphthalene sulphonic acid (0.2% in methanol) followed by viewing under UV. Lipids were identified by comparison with authentic lipid standards and later using spraying reagents.

#### Gas chromatography - mass spectra analysis

The crude lipid extract was transmethylated using 2.0 M KOH in methanol and *n*-heptane according to the method as described (Ichihara et al., 1996) with minor modification. Extracted lipids (10 mg) were dissolved in 2 ml heptane followed by 4 ml of 2.0 M methanolic KOH. The tube was then vortexed for 2 min at room temperature.

After centrifugation at 4000 rpm for 10 min, the heptane layer was taken for GC-MS analyses. The lipid composition was analysed by GC Clarus 500 with autosampler (Perkin Elmer, USA) equipped with a flame ionization detector and a fused silica capillary SGE column (30 m × 0.32 mm, ID × 0.25 µm, BP 20 0.25 UM, USA) coupled to MALDI-TOF (Applied Biosystems, 4800). Various fatty acids were identified by comparing the retention times of various peaks obtained from the mass spectra of the samples.

#### Statistical analysis

Significance of differences between starved and normal tissue total lipids and metabolite concentrations were assessed using a one-way ANOVA with a Tukey's post test, with  $P < 0.05$  considered statistically significant. Results were reported as means ± SD or as percent change from normal control.

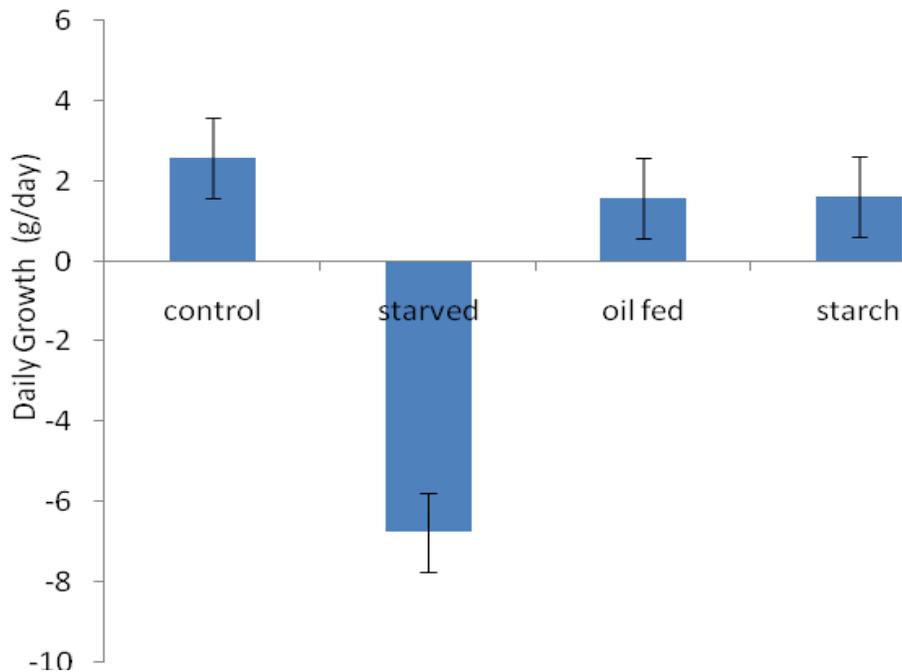
## RESULTS

#### Daily growth and condition factor

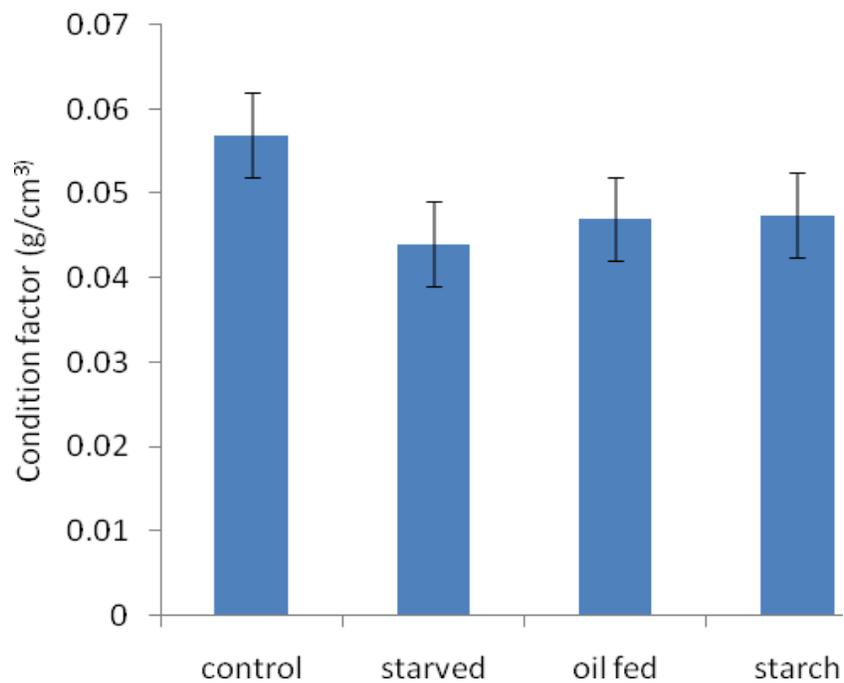
Figure 1 shows the daily growth pattern of differentially fed rats. Control group exhibited a significantly higher daily growth rate in mass per day compared to the other three dietary groups. The control had a mean specific growth rate of 2.565 g/day; oil fed, 1.585 g/day; starch fed, 1.68 g/day and the starved, 6.7 g/day (Figure 1). The control group also exhibited a significantly higher ( $P < 0.05$ ) mean condition factor of (0.058) than estimated mean condition factor of 0.048, 0.047 and 0.042 for starch-fed, oil-fed and starved groups, respectively (Figure 2).

#### Lipid composition

Estimated mean serum lipid triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol concentrations at the end of each diet period are shown in Figure 3. Serum total cholesterol was greater by 0.40 mmol/L in the starved rats (4.30 mmol/L), 0.65mmol/L in the oil fed rats (4.55 mmol/L), 0.75 mmol/L in the starch fed rats (4.65 mmol/L) when compared with the control (3.90 mmol/L), respectively. LDL-cholesterol was greater by 0.9 mmol/L (2.10 mm/L), 1.1 mmol/L (2.3 mmol/L) and 0.95 mmol/L (2.15 mmol/L) in subjects starved, fed with oil, and fed with starch, respectively, when compared with the control (1.2 mmol/L). However, triglyceride was lowered by 1.3, 1.4 and 0.65 mmol/L in starved, oil fed and starch fed rats, respectively, when compared with the control (2.7



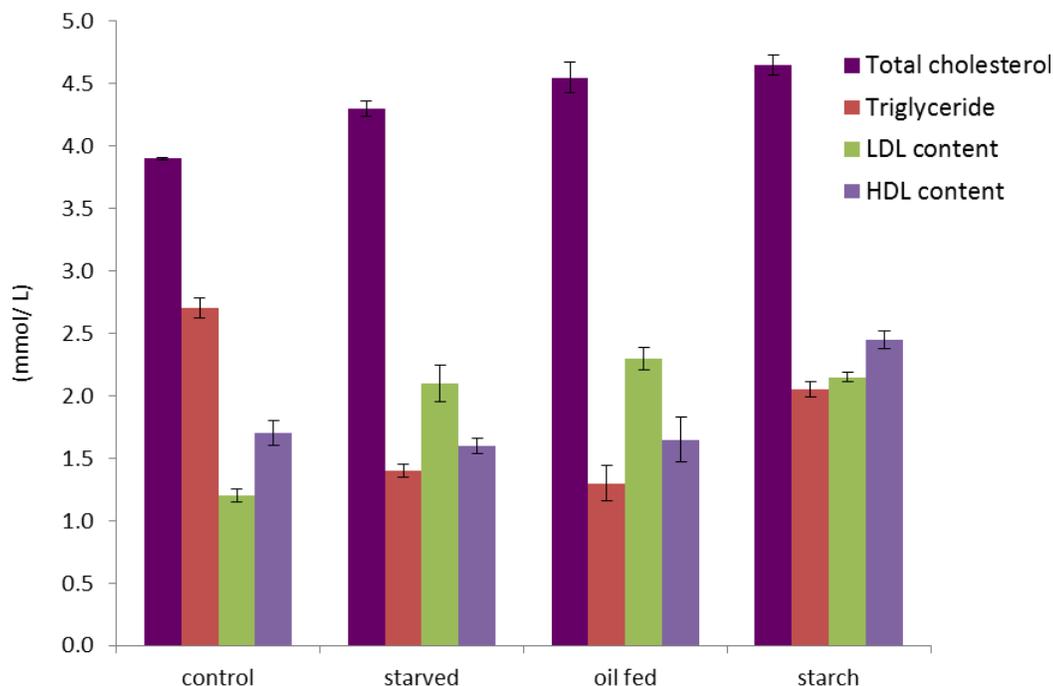
**Figure 1.** Daily growth pattern of the differentially fed rats.



**Figure 2.** Average condition factor of the differentially fed rats.

mmol/L) while HDL-cholesterol was also lowered by 0.1 and 0.15 mmol/L in starved and oil fed groups, respectively, when compared with the control (1.7 mmol/L), but there was an increase by factor of 0.75

mmol/L in the HDL-cholesterol of starch fed group when compared with the control. All differences were significant. The polar lipids that were tentatively identified were designated diphosphatidyl glycerol (DPG), phospho-



**Figure 3.** Serum Lipid profile of the differentially fed rats. Values are means  $\pm$  SD are given in mmol/l.

tidylcholine (PC) phosphatidyl serine (PS) and Cholesterol (CL) in order of decreasing polarity. Neutral lipids were also present in all the four groups and was designated neutral lipids (NL) at the solvent front on the TLC plate (Figure 4). The polar lipids from starch fed rats seemed to be almost identical with that of the lipid enriched diet rat group with significant differences only in the proportion of the components. The polar lipids from the starved rats seemed to be almost identical with that of the control group also with significant differences in the proportion of the components. DPG was much more pronounced in starch fed rats and lipid fed rats when compared with the control group.

#### Gas chromatography - mass spectra (GC/MS) analysis

A typical GC/MS profile of crude lipid extracts of the brain and the liver was analyzed from negative electrospray ionization in the full-scan mode. This approach allowed us to obtain the spectra (Figure not shown). Analysis and interpretation of MS spectra of each ion in the MS spectra allowed for the identification of fatty acyl chain composition of brain and liver.

#### Brain profile

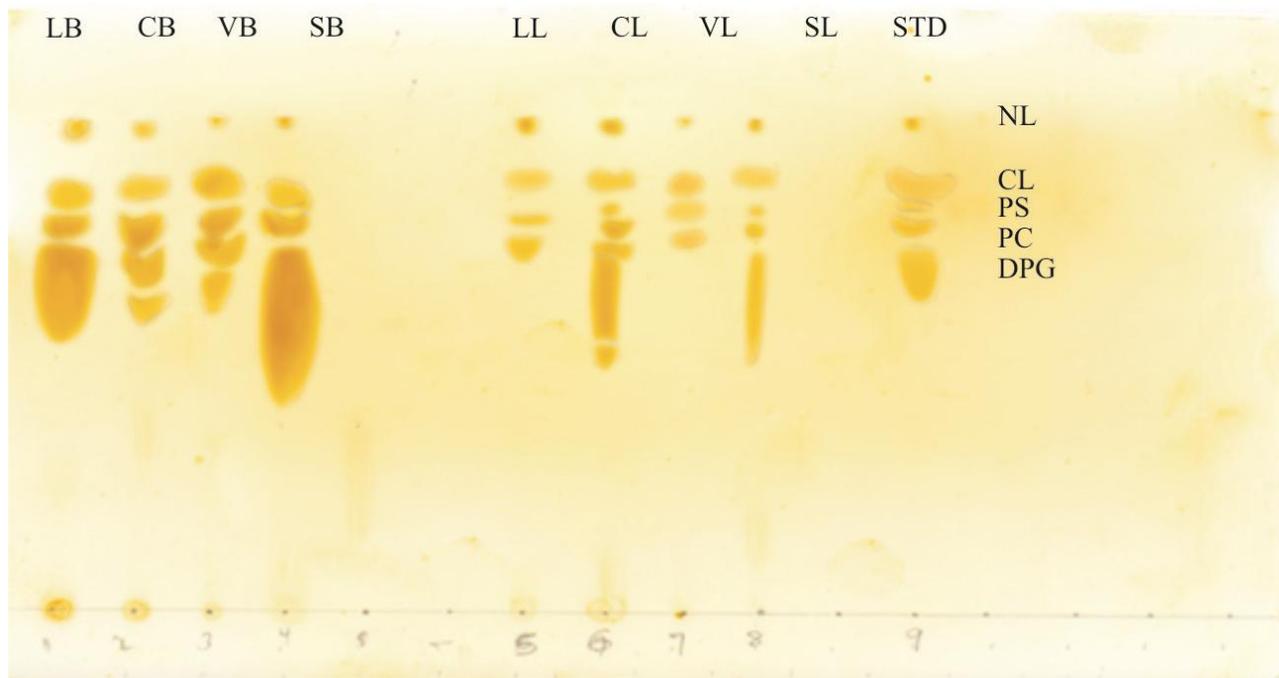
The chromatogram from the GC-mass spectra analyses shows that palmitic acid, oleic, lignoceric, stearic, arachi-

donic acid, docosatetraenoic and docosahexaenoic acid, were present in the control group in different percentages with no traces of lauric acid (Table 2). The starch fed rats also had a similar profile with that of the control except for the complete absence of linoleic acid which made up only 0.61% of the control's fatty acid profile. However, the dominant fatty acid in the starved rat was lauric acid which made up 53.77% of the fatty acid profile of this rat with just traces of oleic acid, docosatetraenoic acid and docosahexaenoic acid. The oil fed group has all the fatty acids present in the control although in varying percentages but also with an additional presence of 8.94% lauric and 1.74% lignoceric acid (Figure 6).

#### Liver profile

The chromatogram revealed the presence of palmitic acid, oleic, stearic, linoleic, arachidonic, behenic, lignoceric, docosatetraenoic and docosahexaenoic acid in varying percentages in the liver of the control (Table 3). The starved group lacked arachidonic and behenic acid but had 7.81% palmitoleic acid when compared with the control. No traces of linoleic, behenic and lignoceric were found in the liver of the oil fed group.

About half of the total fatty acids in the liver of the starch fed group was oleic acid, this group had the highest amount of oleic acid when compared with the control and other groups, it was also the only group that gave the presence of lauric acid, although, it lacked stearic, linoleic, and arachidonic acid when compared



**Figure 4.** The separation of total lipids and lipid fractions from the brain and liver of lipid fed rats, starch fed rats, starved rats, and control group. The solvent was diisobutyl ketone - chloroform - methanol acetic acid - H<sub>2</sub>O (30: 45: 15: 20: 4 v/v). The samples were: 1, Total brain lipids from lipid fed rats; 2, total brain lipids from control group; 3, total brain lipids from starved group; 4, total brain lipids from starch fed rats; 5, total liver lipids from lipid fed rats; 6, total liver lipids from control group; 7, total liver lipids from starved group; 8, total liver lipids from starch fed rats; 9, standard mix standards: DPG, Diphosphatidyl glycerol; PC, phosphatidylcholine; PS, phosphatidyl serine; CL, cholesterol; NL, neutral lipids developed with iodine crystal.

**Table 2.** Distribution of fatty acids in the brain of the selectively fed rats as identified by GC-MS.

Systemic name	Fatty acid	No. of carbon	Control (m%)	Starved (m%)	Oil fed (m%)	Starch fed (m%)
Dodecanoic acid	Lauric acid	12	0±0.0002	53.77±1.34	8.94±0.02	0±0.0001
Hexadecanoic acid	Palmitic acid	16	26.26±1.4	19.89±0.004	21.14±0.1	31.93±0.02
9-Octadecenoic acid	Oleic acid	18	34.33±0.07	5.83±0.003	21.77±0.3	42.05±0.05
9,12-Octadecadienoic acid	Linoleic acid	18	0.61±0.002	0±0.0001	0.53±0.04	0±0.0002
Octadecanoic acid	Stearic acid	18	16.26±0.005	0±0.0002	14.41±0.01	23.14±0.07
5,8,11,14-Eicosatetranoic acid	Arachidonic acid	20	8.44±0.002	0±0.0002	20.48±0.08	2.88±0.001
Tertacosanoic acid	Lignoceric acid	24	0±0.0001	0±0.0001	1.74±0.02	0±0.0001
Docosatetraenoic acid	Adrenic acid	22	11.21±1.2	8.21±0.2	11.24±0.12	18.56±1.55
Docosahexaenoic acid	Cervonic acid	22	10.41±1.01	3.21±0.05	10.05±0.7	9.23±0.96

Data represents mean ± SD of 3 independent experiments P ≤ 0.05 vs. control; Tukey's post-test.

with the control (Figure 5).

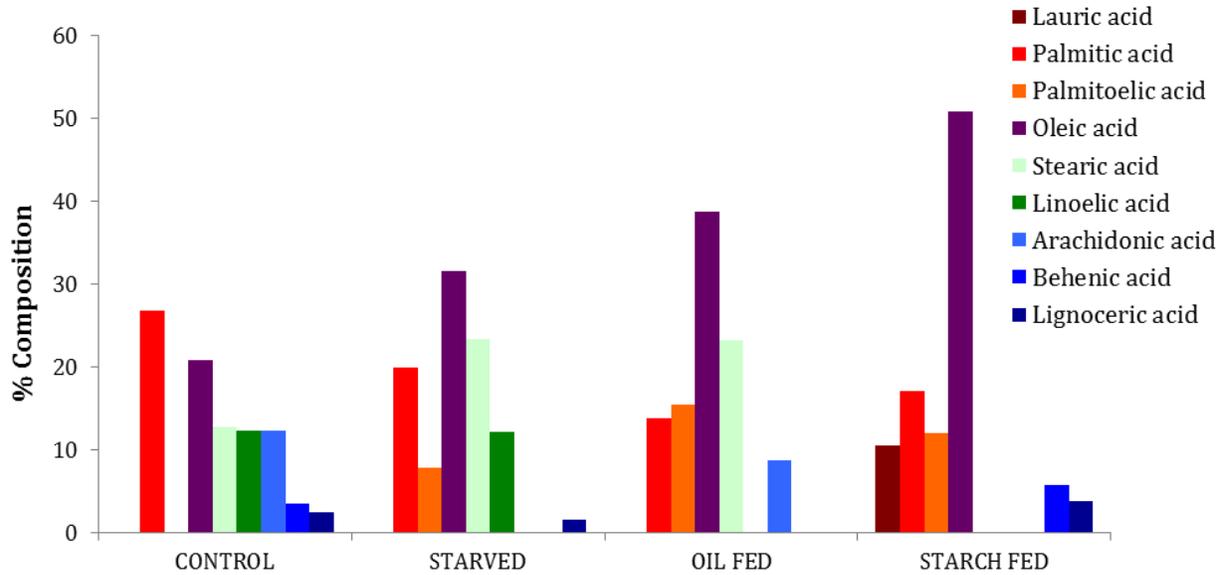
### Sex steroidal hormones

The result of the determination of steroid sex hormones is shown in Figure 7. Estimated mean value of estradiol in the starved, starch fed and oil fed were 539.5, 683.5 and 679.5 pg/ml, respectively, while the control had 710.5 pg/ml. Estimated values of testosterone in the starved, starch fed and oil fed groups were 710, 823 and 1027,

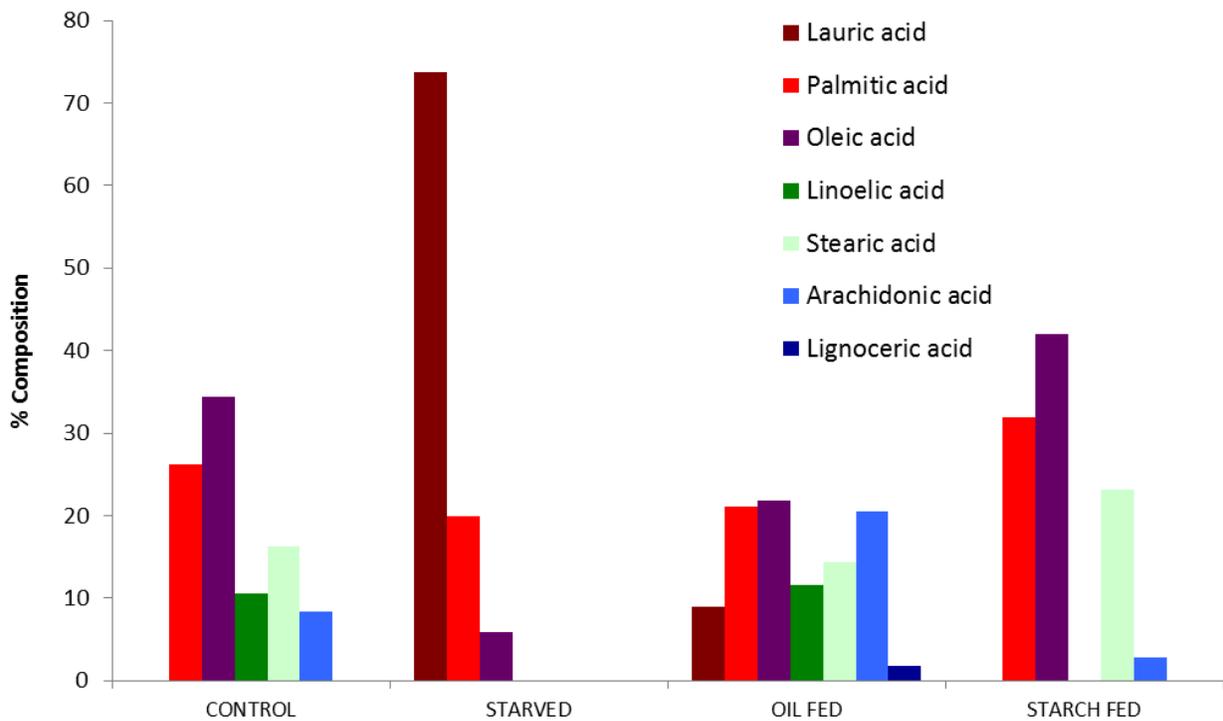
pg/ml respectively, while the control group had estimated testosterone value of 1132.5 pg/ml. Estimated 11-ketotestosterone mean values of the starved, starch fed and oil fed rats were 584, 722.5 and 928.5 pg/ml, respectively, while that of the control was 1120 pg/ml.

### DISCUSSION

The results demonstrate that diet-related changes in the fatty acid composition of the tissues could have signifi-



**Figure 5.** Histogram of the various fatty acids identified by GC-MS in the liver of the rats. Data represents mean  $\pm$  SD of 3 independent experiments  $P \leq 0.05$  vs. control; t test.



**Figure 6.** Histogram of the various fatty acids identified by MS in the brain of the rats. Data represents mean  $\pm$  SD of 3 independent experiments.  $P \leq 0.05$  vs. control; t, test.

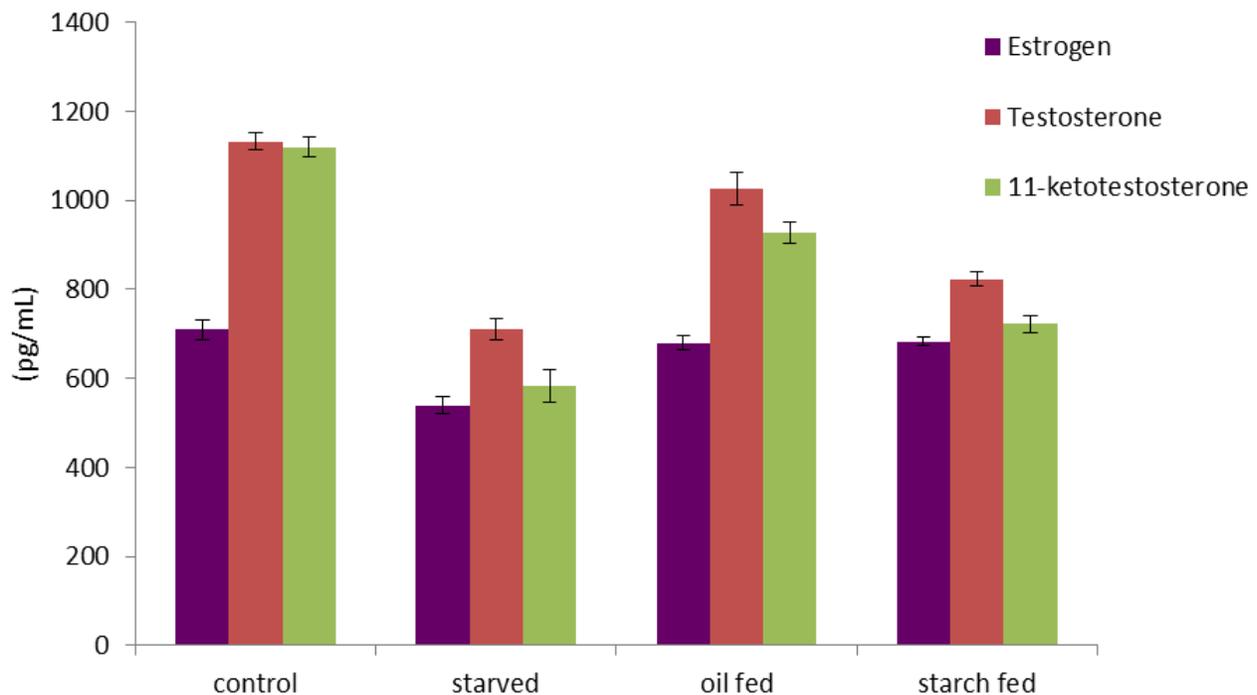
cant effects on major physiological traits of performance and metabolism in rats. The starch fed groups and lipid fed groups revealed a number of associations between the percentage content of specific fatty acid in the

percentage and/or types of fatty acids pools of both brain and liver and particular traits of growth, metabolism and steroidal hormones. It has been well established that nutrition plays a vital role in the etiology of hyperlipide-

**Table 3.** Distribution of fatty acids in the liver of the selectively fed rats as identified by GC-MS.

Systemic name	Fatty acid	No. of carbons	Control (m%)	Starved (m%)	Oil fed (m%)	Starch fed (m%)
Dodecanoic acid	Lauric acid	12	0±0.0002	0±0.0003	0±0.0001	10.46±0.02
Hexadecanoic acid	Palmitic acid	16	26.87±0.02	19.96±0.08	13.75±0.05	17.08±1.01
9-Hexadecenoic acid	Palmitoleic acid	16	0±0.0001	7.81±0.01	15.47±0.02	12.08±0.004
9-Octadecenoic acid	Oleic acid	18	20.8±0.04	31.52±1.02	28.78±0.2	40.12±1.58
Octadecanoic acid	Stearic acid	18	12.81±0.1	23.33±0.1	0.23±0.003	0±0.0004
9,12-Octadecadienoic acid	Linoleic acid	18	12.37±0.003	12.18±0.02	0±0.0001	0±0.0001
5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20	12.36±0.01	0±0.0001	8.76±0.02	0±0.0002
Docosanoic acid	Behenic acid	22	3.54±0.02	0±0.0002	0±0.0001	5.78±0.12
Tetracosanoic acid	Lignoceric acid	24	2.4±0.02	1.59±0.04	0±0.0002	3.78±1.02
Docosatetraenoic acid	Adrenic acid	22	9.21±1.2	2.21±0.2	1.14±0.12	4.52±1.55
Docosahexaenoic acid	Cervonic acid	22	1.42±1.01	1.11±0.05	10.01±0.7	5.03±0.96

Data represents mean ± SD of 3 independent experiments P≤ 0.05 vs. control; Tukey's post-test.



**Figure 7.** Steroid sex hormones of the selectively fed rats. Estradiol, testosterone, and 11-ketotestosterone are decreased in the starved, oil fed and starch fed groups when compared with the control.

mias, impaired cognitive function and alteration in sex steroidal hormones. Several animal and human studies have confirmed the hypercholes-terolemic properties of saturated fatty acids and cholesterol which include increasing total cholesterol and altering lipoprotein pattern and whose mechanism remain under study (Enos et al., 2013). High fat diets are strongly linked with the accumulation of excess body fat, chronic inflammation, and metabolic perturbations, ultimately leading to poorer

health outcomes (Bhargava and Lee, 2012) most of the available supporting literature is limited by the lack of control for various nutrients (for example, protein:carbohydrate:fat among others). We examined the effect of different dietary regimen on lipid profile changes in serum, brain and liver, metabolism. From the results of the daily growth and average condition factor carried out in this study, the control group exhibited a non-significantly higher daily growth rate in mass per day

compared to the other two dietary (starch enriched and soy oil enriched) groups, but there was a significant decline in weight in the group fed with carboxyl methyl cellulose, (starved) when compared with the control. The significant decline in the growth rate of the CMC fed rat might be attributed to unavailability of food for synthesis of building blocks for healthy living. The energy needs of the brain are supplied by metabolism of glucose and oxygen for the phosphorylation of adenosine di phosphate (ADP) to adenosine tri phosphate (ATP). Most of the ATP generated is used in the brain in maintaining intracellular homeostasis and trans- membrane ion gradients of sodium, potassium, and calcium. Energy failure results in rapid loss of ATP and uncontrolled leakage of ions across the cell membrane that results in membrane depolarization (Adibhatla and Hatcher, 2005).

Furthermore, increased total serum cholesterol and LDL-cholesterol in the starved rat is most likely due to the degradation of the energy stores (in form of glucose, amino acids and fats) since the breakdown of these store of energy produces acetyl CoA while releasing energy in form of ATP. This acetyl CoA is a precursor to cholesterol synthesis and it can also be used in the synthesis of new fatty acids but the reduction in the amount of triglyceride noticed in these starved rat and because free unesterified fatty acids are released from triglycerides during fasting to provide a source of energy and of structural components for cells, the possibility of fatty acid synthesis is ruled out in the starved rat, thus, the acetyl CoA produce may have been shuttled to cholesterol synthesis.

Dietary saturated fat intake has been associated with an increased risk of atherosclerotic cardiovascular disease and metabolic diseases, such as obesity and type 2 diabetes. This effect is thought to be mediated by an increase in plasma cholesterol, mainly low-density lipoprotein cholesterol I (Joan et al., 2011). This may further buttress why triglycerides levels are also reduced in the starch fed and lipid fed rats.

However, acetyl CoA is also formed during degradation of carbohydrates, it is understandable how an excess of carbohydrates can result in the formation of fats, which can be kept in the adipose tissue (Tobias and Robert, 2012). This could account for the significant increase in the HDL-cholesterol of the starch fed group. Strong evidence supports a HDL-C-raising effect of diets enriched in saturated fat and cholesterol in humans (Kotseva et al., 2008).

In a well fed animal, the glycerol, fatty acids, mono- and di-glycerides resulting from digestion pass across the intestinal membrane and are resynthesized to triglyceride in the epithelial cells, therefore poor dieting may also account for the reduced levels of triglycerides in the starved, starch fed and lipid fed groups. Also the rate of absorption of the glycerides may differ according to diet composition of ingested lipids. Positive associations between cholesteryl ester transfer protein activity, LDL-cholesterol concentrations were found in animals fed a

diet high in saturated fat. The changes in LDL cholesterol was explained by diet-induced down-regulation of LDL receptor activity through cleavage inhibition of the precursor protein (sterol regulatory element binding protein (Lars et al., 2007). This chain of events would allow for less hepatic clearance of LDL cholesterol. Overall, this relation is of interest, but its mechanism requires further elucidation, particularly during weight loss.

From the results TLC analyses, the polar lipids that were tentatively identified were designated DPG, PC, PS, CL in order of decreasing polarity. Neutral lipids were also present in all the four groups and was designated NL at the solvent front on the TLC plate. The polar lipids from starch fed rats seemed to be almost identical with that of the Lipid group with significant differences only in the proportion of the components. The polar lipids from the starved rats seemed to be almost identical with that of the control group also with significant differences in the proportion of the components. DPG was much more pronounced in starch fed rats and lipid fed rats when compared with the control group. The similarities in the type of lipid identified in all the four groups supported previous reports on the general similarities of membrane and tissue lipids of different cells (Edward et al., 2010).

GC-MS results that show alterations in the feeding pattern of the albino rats has led to a marked imbalance in the ratio of saturated to unsaturated fatty acids in the carbohydrate fed rats, lipid fed rats and CMC fed rats when compared with the control. The major fatty acids produced by the CMC fed rats were the saturated fatty acids; there was only a trace of unsaturated fatty acid in the brain of the rats in this group. It can be inferred that diet may be a major determinant in the balance of saturated to unsaturated fatty acids in animals. Convincing data exist that show the ability of dietary saturated fatty acids to increase LDL cholesterol in animals and humans, also showing how mono-unsaturated fatty acids, carbohydrate and saturated fatty acids influence lipid profiles (Lars et al., 2007).

The marked reduction in unsaturated and poly unsaturated fatty acids in the starved rats could mean that organisms under stress may not be able to produce the essential fatty acids needed for healthy living and reproduction. The marked reduction of the unsaturated and polyunsaturated fatty acids could also be termed as one of the ways by which organisms adapt to stress and starvation. There was also an increase in the amount of polyunsaturated fatty acid in the oil fed when compared with the control. The starch fed group had approximately the same percentage of saturated fatty acid with the control group but the animals had most of the remaining fatty acids in the saturated form and only about 20% of polyunsaturated fatty acids. The differences in the amount of these varying fatty acids could be as a result of adjusting membrane saturation in the brain of these animals. Cognitive impairment is a major component of

dementing syndromes and influences the individual's ability to function independently (Crichton et al., 2010). Alzheimer's disease (AD), the most common form of dementia, is a progressive brain disorder affecting regions that control memory and cognitive functions, gradually destroying a person's memory and ability to learn, reason, communicate and carry out daily activities (Rao and Hatcher, 2007). Altered lipid metabolism is also believed to be a key event which contributes to CNS injury (Adibhatla et al., 2006). Adjusting membrane saturation induced by alteration in feeding pattern may be a contributory factor in the reduction of the cognitive performance of the CMC fed rats when compared to the control. This can also be correlated to results of growth rate and condition factor. High LDL and total cholesterol levels are also associated with cognitive impairment (Tristano et al., 2013).

Based on the fact that nutrition has a significant impact on the listed factors for cognitive dysfunction (for example, cardiac function) (Lemon et al., 2010), poor dietary habits may also contribute to poor cognitive performance. In turn, poor diet (for example, lack of fruits and vegetables, high fat and sodium intake) has been linked with reduced cognitive function among persons with medical illnesses that are frequently comorbid with heart failure. For instance, lack of fruits and vegetables, reduced adherence to Mediterranean diets, and diets high in fat intake have been shown to reduced cognitive functioning in vascular dementia, type 2 diabetes, Alzheimer's disease, and mild cognitive impairment (Crichton et al., 2010; Devore et al., 2009; Barberger et al., 2007).

The liver was observed to have appreciable more fatty acids than the brain, this may be due to the central role the liver plays in fatty acid metabolism. Next to adipose tissue, liver has the greatest capacity to store lipids in lipid droplets (Tobias and Robert, 2012). The adaptation of the liver to the periods following meals and the periods of fasting may be easy to upset by nutrition. Lauric acid, which is a 12-carbon straight chain fatty acid, was observed to be the predominant fatty acid in the brain of the starved rat as opposed to the complete absence in the control. This may be as a result of a breakdown by  $\beta$ -oxidation of the longer chain and unsaturated fatty acids that may have been stored in the starved rats. The predominance of lauric acid may also be as a result of the body scavenging for the available unsaturated and longer chain fatty acids from the animal in order to break it down and generate energy since fats are high-energy stores (Tobias and Robert, 2012). Linoleic acid which is a precursor to the synthesis of arachidonic acid has been found to be completely absent also in the brain of the starved rat, linoleic and arachidonic acid have been implicated during early brain development as well as during the development of late life cognitive decline and dementia (Innis, 2008; Oken et al., 2008; Boudrault et al., 2009; Das, 2008) since animals are expected to get linoleic acid from the diet (Muldoon et al., 2010); it is not

surprising that the starved animal lacked this essential fatty acid. The starved animals were observed to be lean and wrinkled; this skin trouble may have been as a result of a deficiency in the essential fatty acids. Estrogen, testosterone, and 11-ketotestosterone were decreased in the starved, oil fed and starch fed groups when compared with the control, but the marked decrease was more pronounced in the starved animal. The reduction of unsaturated fatty acids may be linked to this reduction in sex steroidal hormones since vitamin E deficiency has been observed in certain people suffering from congenital problems of fat absorption and since the requirement in man is a function of diet (Tobias and Robert, 2012; Vaisman et al., 2008).

Sex steroid hormones such as estradiol-17 $\beta$  and testosterone are particularly important in regulating the development and function of reproductive activity in mammals (Gallagher et al., 2001). In addition, 11-ketotestosterone is considered to be a dominant androgen in males and responsible for the development of the testis and sexual behavior (Cuisset et al., 2011). Due to the importance in reproduction and the fact their level can be quantitated in the plasma of the vertebrates, sex steroid profile can be used as biomarkers of altered-feeding induced reproductive stress. Although measure of plasma concentrations of sex steroids indicates the overall effects on circulating steroid concentration, they do not provide information as to the mechanism underlying how different feeding pattern exerts their effects. Hormones alteration can be caused by a number of factors, including changes in hormone synthesis, secretion, metabolism, hepatic catabolism or binding to plasma proteins. In general, increased in plasma steroid in others over the starved group can be the result of increased steroid biosynthesis or decreased hepatic catabolism by biotransformation enzymes. In sum, feeding rats on a diet rich in soy oil and carbohydrate for a 14-day period produced marked alterations in lipid profile in serum and tissues, as well as modifications in steroidal hormones.

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Full Length Research Paper

## Anti-motility and reductions in the concentrations of gut electrolytes: Mechanisms for the anti-spasmodic use of the seeds of avocado (*Persea americana* Mill) in folk medicine

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The seeds of avocado (*Persea americana* Mill) are used in traditional medicine to treat, allay or prevent some spasm-related disorders, for instance, diarrhoea. The chloroform and methanol fractions of the chloroform-methanol extract of the seeds of *P. americana* were investigated for their qualitative and quantitative phytochemical constituents as well as effects on gastro-intestinal motility (transit) and castor oil-induced intestinal fluid sodium ion (Na<sup>+</sup>) and potassium ion (K<sup>+</sup>) concentrations in Wistar rats. The qualitative and quantitative phytochemical studies of the chloroform and methanol fractions showed the presence and amounts of alkaloids (2.92 ± 0.14 g/100 g and 2.81 ± 0.08 g/100 g respectively), flavonoids (3.43 ± 0.19 g/100 g and 3.11 ± 0.16 g/100 g, respectively), tannins (2.64 ± 0.13 g/100 g and 2.85 ± 0.14 g/100 g, respectively), steroids (1.51 ± 0.07 g/100 g and 1.27 ± 0.04 g/100 g, respectively), saponins (2.35 ± 0.08 % and 2.47 ± 0.09%, respectively), terpenoids, proteins and carbohydrates in both fractions. Fats and oil were present only in the chloroform fraction. At the two doses (100 and 200 mg/kg body weight), the chloroform and methanol fractions produced significant (p<0.05) and dose-related decreases in the gastro-intestinal motility and concentration of the intestinal fluid potassium ions but only the chloroform fraction at the dose of 200 mg/kg body weight significantly (p<0.05) decreased the concentration of the intestinal fluid sodium ions. Results of the fractions were comparable with those of the standard anti-diarrhoeal drug, hyoscine butylbromide (3 mg/kg body weight). The results indicate that the chloroform-methanol extract of the seeds of *P. americana* contains compounds with anti-spasmodic effect.

**Key words:** *Persea americana*, spasm-related, castor oil, gastro-intestinal motility and electrolytes.

### INTRODUCTION

Diarrhoea is characterised by increased frequency of bowel movement, wet stool and abdominal pain (Nitinkumar et al., 2010). Diarrhoea remains one of the commonest illnesses of children and one of the major

causes of infant and childhood mortality in developing countries. It is estimated that 3.3 million deaths occur each year among children under five year old. In Nigeria, diarrhoeal infection remains the number one killer disease

among children under the age of five, while 7-12 month old babies remain the most susceptible (Sule et al., 2009). Nigeria, the fourth largest economy in Africa with an estimated per capita income of \$350 has over half of its population living in poverty (WHO, 2007). This implies that not very many persons can afford orthodox medicine in curing diseases. In addition, many synthetic chemicals like diphenoxylate, loperamide and antibiotics are available for the treatment of diarrhoea but they have some side effects (Nitinkumar et al., 2010). Thus, the search for safe and more effective agents has continued to be a vital area of active research. Since ancient times, diarrhoea has been treated orally with several medicinal plants or their extracts based on folklore medicine.

*Persea americana* (avocado) is an evergreen tree belonging to the laurel family, Lauraceae. Various parts of the plant have been shown to possess medicinal properties. The edible fruit pulp contains up to 33% oil rich in monounsaturated fatty acids that are believed to modify the fatty acid contents in cardiac and renal membranes and enhance the absorption of alpha/beta-carotene and lutein (Salazar et al., 2005). The carotenoid content has been reported to play significant role in cancer risk reduction (Lu et al., 2005). Other properties of the oil include wound-healing and hepatoprotection (Nayak et al., 2008). The aqueous leaf extract has analgesic and anti-inflammatory activities (Adeyemi et al., 2002). The seeds of *P. americana* have diverse applications in ethno-medicine, ranging from treatment for diarrhoea, dysentery, toothache, intestinal parasites, skin treatment and beautification (Alhassan et al., 2012). In this study, we report the phytochemical and anti-spasmodic activity of the chloroform-methanol extract of the seeds of *P. americana* in normal and castor oil-induced diarrhoeal rats.

## MATERIALS AND METHODS

### Plant

Fresh fruit of *P. americana* were harvested from trees at various points in Iheapku-Awka, Igbo Eze South Local Government Area of Enugu State, Nigeria. The fruit seeds were identified by Prof. (Mrs.) May Nwosu of the Department of Botany, University of Nigeria, Nsukka.

### Preparation of the extract

The fresh fruit were split open with a knife and the seeds removed. The seeds were washed with distilled water and sliced with knife. The sliced seeds were spread on a clean mat in a well-ventilated room with regular turning to enhance even drying and avoid decaying. The sliced seeds were shade-dried for 8 weeks. The shade-dried sliced seeds were milled with an electric blender and 1380 g of the milled seeds was macerated in 5 volumes (w/v) of chloroform-methanol (2:1) for 24 h. The mixture was separated with Whatman No. 1 filter paper. The filtrate of the macerate was shaken with distilled water that measured 20% its volume to obtain two fractions. The upper fraction (methanol fraction) was separated

from the lower fraction (chloroform fraction). The methanol and the chloroform fractions were concentrated in a rotary evaporator, dried in a boiling water bath and weighed.

### Phytochemical analyses

Qualitative phytochemical analyses were carried out on both the methanol and the chloroform fractions according to the procedures outlined by Harborne (1998) and Trease and Evans (1989). Quantitative phytochemical analyses were carried out to determine the concentration of the following: alkaloids and flavonoids by the methods of Harborne (1998); tannins by the method of Swain (1979); steroids by the method of Okeke and Elekwa (2003) and saponins by the method of Obadoni and Ochuko (2001).

### Animals

Adult male Wistar rats of between 8 and 12 weeks old with average weight of  $125 \pm 25$  g were obtained from the Animal house of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. The rats were acclimatised for one week under a standard environmental condition with a 12 h light and dark cycle and maintained on a regular feed and water *ad libitum*. The Principles of Laboratory Animal Care were adhered to. The experimental protocol was approved by the University Animal Research Ethical Committee.

### Chemicals and reagents

The chemicals used for this study were of analytical grade. They included the following: hyoscine butylbromide [standard anti-diarrhoeal drug (Sigma-Aldrich, Inc., St. Louis, USA)], methanol and chloroform, 45% (v/v) ethanol (BDH Chemicals Ltd., Poole, England), dilute tetraoxosulphate (VI) acid, 2% (v/v) hydrochloric acid, 1% (w/v) picric acid, methyl orange, activated charcoal, gum acacia, castor oil (laxative), 3% (v/v) tween 80, Dragendorff's reagent, Mayer's reagent, Wagner's reagent, Millon's reagent, Fehling's solution, 5% (w/v) ferric chloride solution, aluminium chloride solution, lead subacetate solution, ammonium solution, Molisch's reagent, filtrate reagent, acid reagent, sodium colour reagent, sodium standard, potassium reagent and potassium standard.

### Gastro-intestinal motility test

Gastro-intestinal motility was evaluated using the method of Mascolo et al. (1994) with little modification.

### Determination of the concentrations of sodium and potassium ions

The concentrations of sodium and potassium ions were determined by the method of Tietz (1994).

### Statistical analysis

The data obtained from the laboratory were subjected to One Way Analysis of Variance (ANOVA). Significant differences were observed at  $p \leq 0.05$ . The results were expressed as means of five replicates  $\pm$  standard deviations (SD). Analysis was performed using Statistical Package for Social Sciences (SPSS), version 16.

**Table 1.** Qualitative phytochemical constituents of the chloroform and the methanol fractions.

Phytochemical constituent	Chloroform fraction	Methanol fraction
Alkaloids	++	+
Flavonoids	+++	++
Glycosides	ND	+
Proteins	+	+
Carbohydrates	+	+
Reducing sugars	ND	ND
Saponins	+++	+++
Tannins	+++	++
Fats and oil	+	ND
Resins	ND	ND
Terpenoids	++	++
Steroids	++	++
Acidic compounds	ND	ND

ND, Not detected; +, present in low concentration; ++ = present in moderately high concentration; +++ = present in very high concentration.

**Table 2.** Amounts of alkaloids, flavonoids, tannins, steroids and saponins in the chloroform and the methanol fractions of *P.americana* seeds.

Phytochemical constituent	Chloroform fraction	Methanol fraction
Alkaloids (g/100 g)	2.92 ± 0.14	2.81 ± 0.08
Flavonoids (g/100 g)	3.43 ± 0.19	3.11 ± 0.16
Tannins (g/100 g)	2.85 ± 0.14	2.64 ± 0.13
Steroids (g/100 g)	1.51 ± 0.07	1.27 ± 0.04
Saponins (%)	2.35 ± 0.08	2.47 ± 0.09

Values are expressed as means of five determinations ± SD.

## RESULTS

### The qualitative and quantitative phytochemical composition of the chloroform and the methanol fractions

The chloroform fraction of the extract contained higher amounts of alkaloids (2.92 ± 0.14 g/100 g), flavonoids (3.43 ± 0.19 g/100 g), tannins (2.85 ± 0.14 g/100 g) and steroids (1.51 ± 0.07 g/100 g) than the methanol fraction while the methanol fraction contained higher amount of saponins (2.47 ± 0.09 %) than the chloroform fraction (Tables 1 and 2).

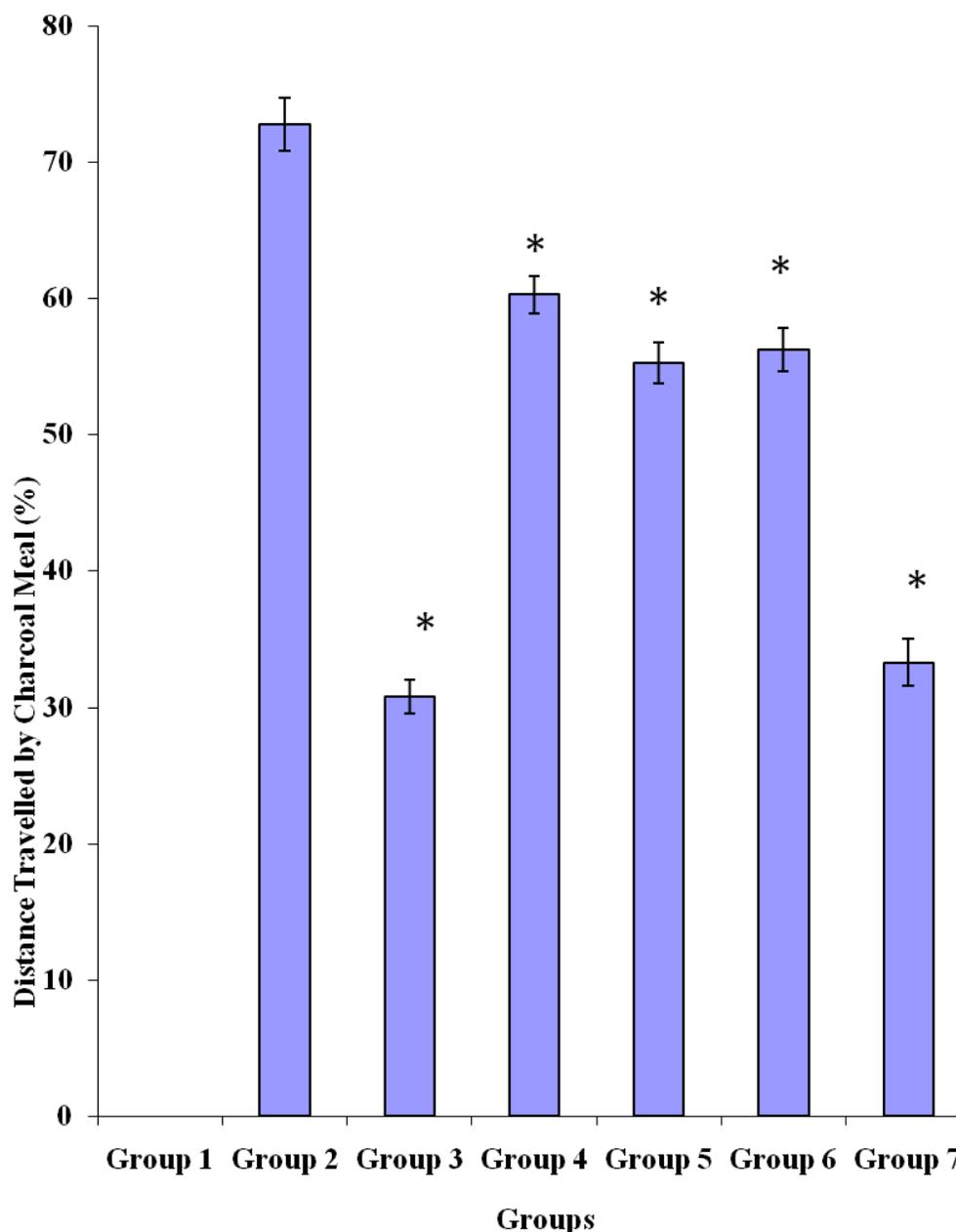
### Effects of the methanol and the chloroform fractions on gastro-intestinal motility

The charcoal meal (gastro-intestinal motility) test was used to determine the propulsive movement along the gastro-intestinal tract (GIT) of rats. As shown in Figure 1, the methanol and the chloroform fractions of the extract at the tested doses (100 and 200 mg/kg body weight of each) significantly ( $p < 0.05$ ) decreased the percentage

distance travelled by the charcoal meal along the gastro-intestinal tract of rats in groups 4, 5, 6 and 7 when compared to the value obtained for rats in the charcoal meal-treated control group (group 2). The observed effects were dose-dependent with percentage distance travelled by charcoal meal as 60.25 ± 1.37, 55.25 ± 1.50, 56.25 ± 1.61 and 33.25 ± 1.75 for rats in the 100 and 200 mg/kg body weight of the methanol fraction-treated groups (groups 4 and 5), 100 and 200 mg/kg body weight of the chloroform fraction-treated groups (groups 6 and 7) respectively, when compared to the value (72.75 ± 1.98) obtained for rats in the charcoal meal-treated control group (group 2). The effects of the methanol and the chloroform fractions of the extract at the tested doses were comparable to that of the standard anti-diarrhoeal agent (hyoscine butylbromide) as shown in Figure 1.

### Effects of the methanol and chloroform fractions on the intestinal fluid sodium ion (Na<sup>+</sup>) concentration

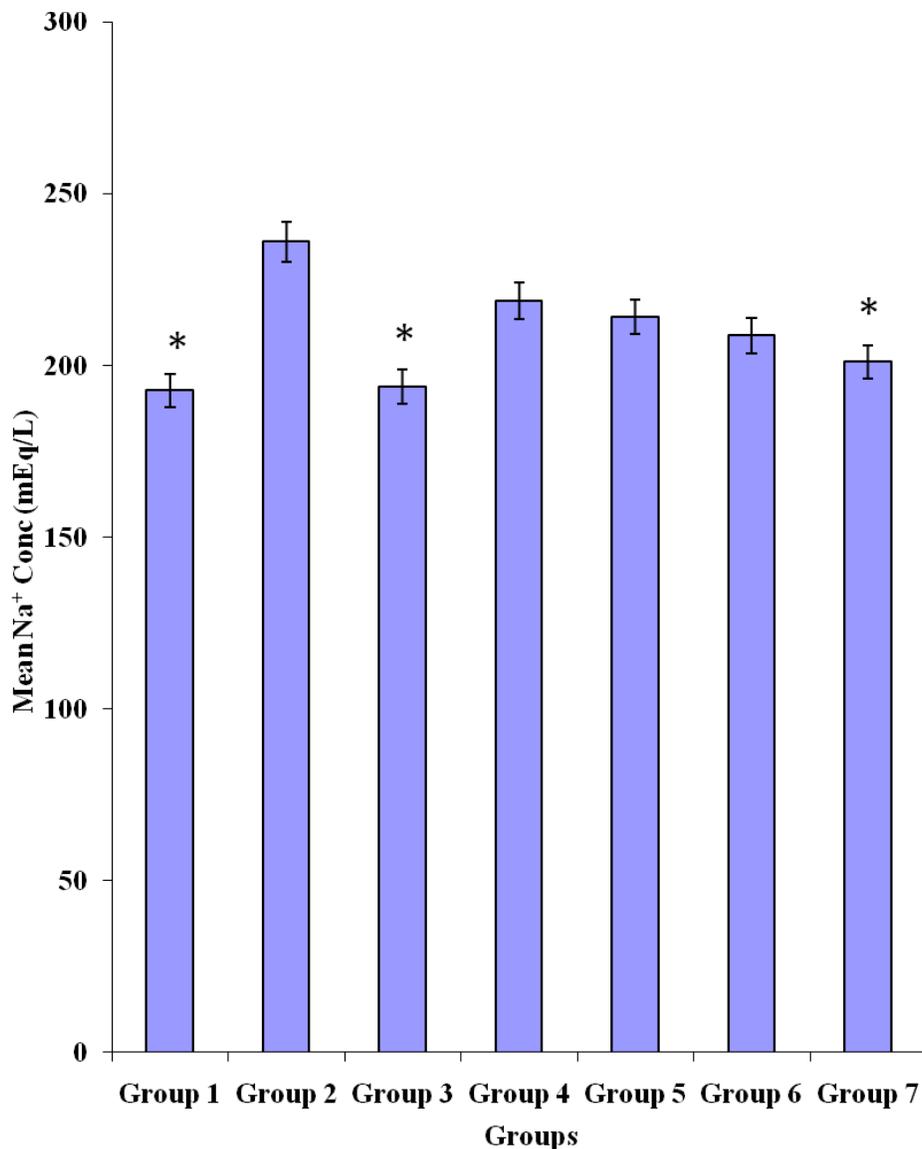
Result of the intestinal fluid sodium ion concentration test as shown in Figure 2, shows that the rats of the castor



**Figure 1.** Effects of the methanol and the chloroform fractions of the chloroform-methanol seed extract of *Persea americana* on the gastro-intestinal motility. Data represented as mean  $\pm$  SD; \*, significantly ( $p < 0.05$ ) lower compared to that of group 2). Group 1, 5 ml/kg b.w of 3% v/v tween 80 (vehicle); group 2, vehicle + 1 ml of castor oil (CO); group 3, 3 mg/kg b.w of hyoscine + 1ml of CO; group 4=100mg/kg b.w of methanol fraction of *P. americana* +1ml of CO; group 5=200 mg/kg b.w of methanol fraction of *P. americana* +1 ml of CO; group 6, 100 mg/kg b.w of chloroform fraction of *P. americana* +1 ml of CO; group 7, 200 mg/kg b.w chloroform fraction of *P. americana* +1 ml of CO.

oil-treated control group (group 2) had significantly ( $p < 0.05$ ) increased intestinal fluid sodium ion concentration ( $236.00 \pm 5.79$ ) when compared to the value ( $192.75 \pm 4.82$ ) obtained for rats in the group that received only the vehicle (group 1). The chloroform fraction of the extract at the dose of 200 mg/kg body

weight, like the standard anti-diarrhoeal agent (hyoscine butylbromide), caused a significant ( $p < 0.05$ ) reduction in the intestinal fluid sodium ion concentration of rats in group 7 ( $201.00 \pm 4.90$ ) when compared to the value ( $236.00 \pm 5.79$ ) obtained for rats in the castor oil-treated control group.

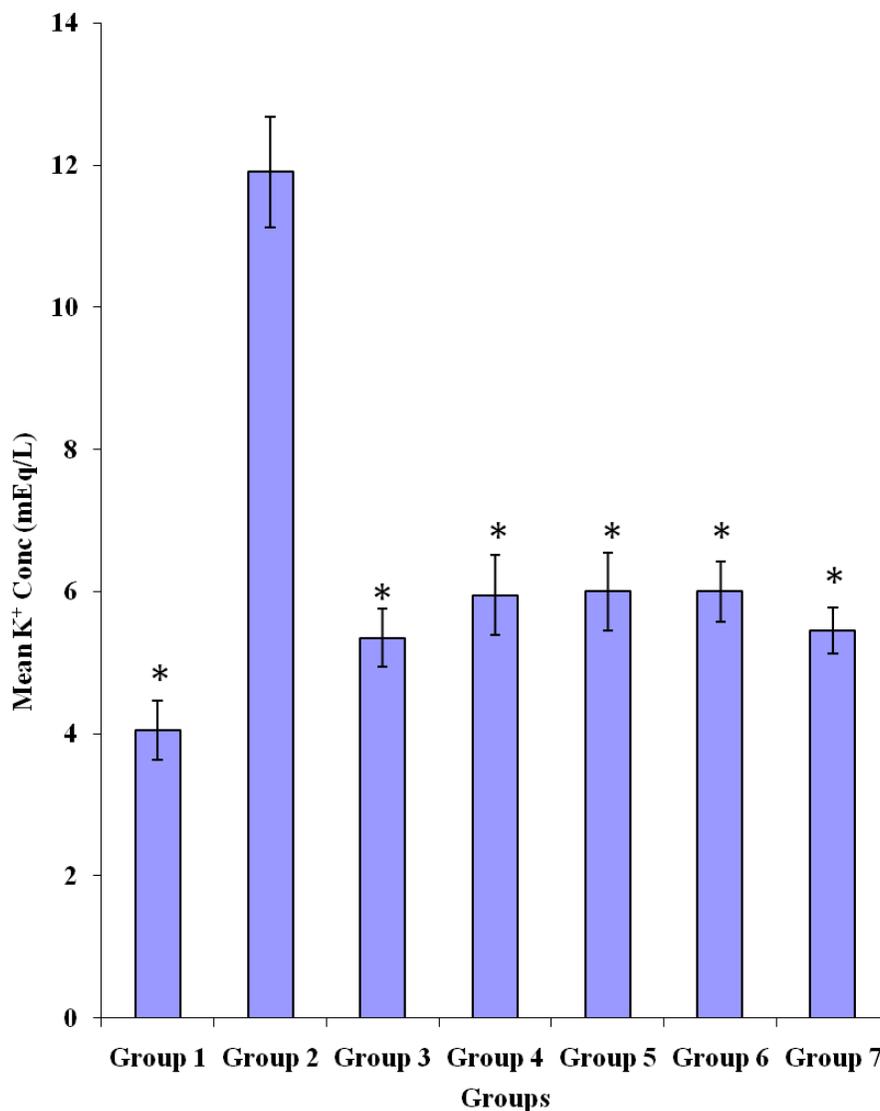


**Figure 2.** Effects of the methanol and chloroform fractions of the chloroform-methanol seed extract of *Persea americana* on intestinal sodium ion (Na<sup>+</sup>) concentration. Data represented as mean  $\pm$  SD; \*, significantly ( $p < 0.05$ ) lower compared to that of group 2. Group 1, 5 ml/kg b.w of 3% v/v tween 80 (vehicle); group 2, vehicle + 1 ml of castor oil (CO); group 3, 3 mg/kg b.w of hyoscine + 1 ml of CO; group 4=100mg/kg b.w of methanol fraction of *P. americana* +1ml of CO; group 5=200 mg/kg b.w of methanol fraction of *P. americana* +1 ml of CO; group 6, 100 mg/kg b.w of chloroform fraction of *P. americana* +1 ml of CO; group 7, 200 mg/kg b.w chloroform fraction of *P. americana* +1 ml of CO.

#### Effects of the methanol and chloroform fractions on the intestinal fluid potassium ion (K<sup>+</sup>) concentration

As shown in Figure 3, the methanol and the chloroform fractions of the extract at the tested doses (100 and 200 mg/kg body weight of each) significantly ( $p < 0.05$ ) reduced the intestinal fluid potassium ion concentration of rats in groups 4, 5, 6 and 7 when compared to that of the rats in the castor oil-treated control group (group 2). The

effects observed were dose-related with the intestinal fluid potassium ion concentration as  $5.95 \pm 0.56$ ,  $6.00 \pm 0.55$ ,  $6.00 \pm 0.42$  and  $5.45 \pm 0.32$  for rats in the 100 and 200 mg/kg body weight of the methanol fraction-treated groups (groups 4 and 5), 100 and 200 mg/kg body weight of the chloroform fraction-treated groups (groups 6 and 7) respectively, when compared to the value ( $11.90 \pm 0.78$ ) obtained for rats in the castor oil-treated control group (group 2). The effects of the methanol and the chloroform



**Figure 3.** Effects of the methanol and chloroform fractions of the chloroform-methanol seed extract of *Persea americana* on intestinal potassium ion (K<sup>+</sup>) concentration. Data represented as mean  $\pm$  SD; \*, significantly ( $p < 0.05$ ) lower compared to that of group 2. Group 1, 5 ml/kg b.w of 3% v/v tween 80 (vehicle); group 2, vehicle + 1 ml of castor oil (CO); group 3, 3 mg/kg b.w of hyoscine + 1 ml of CO; group 4=100mg/kg b.w of methanol fraction of *P. americana* +1ml of CO; group 5=200 mg/kg b.w of methanol fraction of *P. americana* +1 ml of CO; group 6, 100 mg/kg b.w of chloroform fraction of *P. americana* +1 ml of CO; group 7, 200 mg/kg b.w chloroform fraction of *P. americana* + 1 ml of CO.

fractions of the extract at the tested doses were comparable to that of the standard anti-diarrhoeal agent (hyoscine butylbromide) as shown in Figure 3.

## DISCUSSION

The results of the qualitative and quantitative phytochemical analyses of the chloroform and the methanol fractions of the chloroform-methanol extract of

the seeds of *P. americana* showed, in both fractions of the extract, the presence and amounts of alkaloids ( $2.92 \pm 0.14$  and  $2.81 \pm 0.08$  g/100 g in the chloroform and the methanol fractions, respectively), flavonoids ( $3.43 \pm 0.19$  and  $3.11 \pm 0.16$  g/100 g in the chloroform and the methanol fractions, respectively), tannins ( $2.85 \pm 0.14$  and  $2.64 \pm 0.13$  g/100 g in the chloroform and the methanol fractions, respectively), steroids ( $1.51 \pm 0.07$  and  $1.27 \pm 0.04$  g/100 g in the chloroform and the methanol fractions, respectively) and saponins ( $2.35 \pm$

0.08 and  $2.47 \pm 0.09\%$  in the chloroform and the methanol fractions, respectively). This indicates that the bioactive constituents present in the chloroform-methanol extract of the seeds of *P. americana* resided more in the chloroform fraction than in the methanol fraction. Reducing sugars, resins and acidic compounds were found to be absent in both fractions of the extract. The anti-diarrhoeal effect of both fractions of the extract shown in the present study could be, in part, due to the presence of tannins, alkaloids, saponins, flavonoids and steroids. In other words, it is possible that flavonoids and steroids, acting dually or in combination with other phytochemicals, produced the observed anti-diarrhoeal effect of both fractions of the chloroform-methanol extract of the seeds of *P. americana*. Previous studies showed that anti-dysenteric and anti-diarrhoeal properties of medicinal plants were mostly due to tannins, alkaloids, saponins, flavonoids, sterol and triterpenes (Longanga et al., 2000). While flavonoids are known to inhibit intestinal hyper-motility and hydroelectrolytic secretion, tannins denature proteins in the intestinal mucosa by forming protein tannates which make intestinal mucosa more resistant to chemical alteration and reduce secretion (Havagiray et al., 2004). Also, extracts of plants that contain flavonoids are known to modify the production of cyclo-oxygenase 1 and 2 (COX-1 and COX-2) and lipoxygenase (LOX) thereby inhibiting the production of prostaglandins (Sule et al., 2009). Steroids are also useful for the treatment of diarrhoea and may also enhance intestinal absorption of sodium ion ( $\text{Na}^+$ ) and water (Anup et al., 2007).

Anti-motility along the GIT was demonstrated by both fractions of the chloroform-methanol extract of the seeds of *P. americana* as there was dose-dependent reduction in the percentage distance travelled by the charcoal meal along the GIT in the charcoal meal-treated rats. Pre-treatment with both fractions of the extract suppressed the propulsive movement of the charcoal meal as observed by the decrease in the motility of charcoal meal along the GIT. Suppression of the propulsive movement of the charcoal meal along the GIT by both fractions of the extract at least, in part, indicates an anti-diarrhoeal effect of the seeds of *P. americana*. This might be due to the ability of both fractions to reduce peristaltic activity and ultimately bring about a reduction in the gastro-intestinal motility. Decrease in intestinal motility might have led to increased re-absorption of water and electrolytes from faeces and additionally, might have contributed to the reduction in the watery texture of the faeces. It is also possible that both fractions suppressed the propulsive movement of the charcoal meal along the GIT by anti-cholinergic mechanism in a manner similar to the action of the standard anti-diarrhoeal drug, hyoscine butylbromide. This is in agreement with the finding of Sule et al. (2009) who reported that anti-diarrhoeal agents increase intestinal transit time by anti-cholinergic effect.

Study of the effects of both fractions of the chloroform-methanol extract of the seeds of *P. americana* on intestinal fluid sodium ion ( $\text{Na}^+$ ) and potassium ion ( $\text{K}^+$ ) concentrations showed that both fractions of the extract markedly and dose-dependently caused reductions in the concentrations of these electrolytes. These observed effects in part, imply that the seeds of *P. americana* possess anti-diarrhoeal effect. The anti-diarrhoeal effect evidenced here, might be due to the fact that both fractions of the extract probably enhanced the absorption of the electrolytes from the intestinal lumen, while suppressing the rate of their secretion into the small intestine. It has been shown that castor oil causes motility and secretory diarrhoea (Gerald et al., 2007). This is achieved through its dual effects on gastro-intestinal motility as well as transport of water and electrolytes (decreasing  $\text{Na}^+$  and  $\text{K}^+$  absorption) across the intestinal mucosa (Rouf et al., 2003).

In conclusion, the present experimental findings thus, justify the use of the seeds of *P. americana* as an anti-spasmodic agent by the traditional medicine practitioners.

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Full Length Research Paper

## Economic feasibility of on-farm fuel ethanol production from cassava tubers in rural communities

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A simple process for on-farm bioethanol production from cassava, using cassava *koji* supplemented with crude liquid enzyme and yeast was described. On a small scale, a fed-batch mode where 4 kg of *koji*, 2 kg of gelatinized cassava flour and 30 g of yeast cells were mixed and allowed to ferment for two days, followed by addition of 1.5 kg of cassava flour and fermenting for another three days, gave higher ethanol concentration of 7.05% (0.34 g-ethanol/g-cassava flour) than when 3.5 kg of gelatinized cassava flour, 4 kg of *koji* and 30 g of yeast cells were mixed at the same time and allowed to ferment for five days. The process was scaled up 100 times and economic feasibility was evaluated. The total investment cost was seven million, five hundred thousand Nigerian naira (₦) (US\$46,875). With a payback period of five years, the cost of cassava tubers represented 71.73% of the total production cost. At a market price of fresh cassava tubers of ₦10,000/ton, the ethanol production cost was ₦102.5/l (US\$0.641/l), which is not profitable considering the current market price of ethanol (US\$0.597-0.748/l). The process becomes profitable only when the price of fresh cassava tuber is reduced to ₦5,000/ton (US\$31.25/ton). At this price, the ethanol production cost would be ₦58.53/l (US\$0.366/l). The process is recommended for vertically integrated system (on-farm process) where the cassava produced in the farm is used, thereby shielding it from high and fluctuating market prices of cassava.

**Key words:** Fuel ethanol, bioenergy, *koji*, economic analysis, cassava ethanol.

### INTRODUCTION

Due to the non-renewable nature of fossil fuels and various environmental problems associated with their exploration, production and use, a lot of efforts are being made to develop renewable and environmentally friendly bio-energies (Ogbonna et al., 2001; Zhang et al., 2003). Among the various bio-energies, fuel ethanol is already commercially produced in many countries where it is used as octane enhancer, blended with gasoline in various ratios to produce gasohol or used directly in specially designed ethanol engines. Gasohol containing less than 10% ethanol (E10) can be used in most engines without modifications. A major advantage of bio-ethanol is that the feedstock (agricultural materials) is varied, renewable and can be produced in many places.

Most African countries have large areas of fertile land that is not currently used for production of food crops (World Bank Report, 2013). Although opinion vary as to whether such land should be used for energy crop production, with good policy on land use, lands not currently used for food production can be used to grow energy crops. With appropriate technologies and economically viable processes, ethanol production from agricultural biomass would not only help to encourage farming in these countries but will also be a source of foreign earning. Unfortunately, there are only a few commercial fuel ethanol production plants in the continent. South Africa is the only African country within the top 15 ethanol-producing countries in the world

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(Sánchez and Cardona, 2008). This is partly because there is no simple fuel ethanol production technology that can be operated in rural areas without some basic infrastructures.

Raw materials investigated or already used for commercial production of fuel ethanol include sugar crops such as sugar cane, sugar beet, and sweet sorghum (Worley et al., 1992; Sheoran et al., 1998; Ergun and Muttu, 2000; Ogbonna, 2004); cereals such as corn, millet, and sorghum (Sree et al., 1999; Nam et al., 1988; Montesinos and Navarro, 2000); root crops such as potatoes, sweet potatoes, sago, and cassava (Abouzied and Reddy, 1986; Amutha and Gunasekaran, 2001; Roble et al., 2003; Dai et al., 2006); molasses, as well as various types of lignocellulosic materials (Sun and Cheng, 2002; Lau and Dale, 2009; El-Zawaway et al., 2011; Balat, 2011).

The choice of feedstock is very crucial in commercial production of fuel ethanol since the cost of feedstock makes up a significant percentage of the total production cost. Technological issues such as easy with which the material can be converted to ethanol, the ethanol yield and productivity are taken into consideration in the choice of the feedstock for fuel ethanol production. The choice of the feedstock depends on the suitability of available land for their production, the productivity of the crop, the production cost (requirement for fertilizers, and other agrochemicals), environmental sustainability of the crop, social factors (food use and the effects on the prices of food), and on the whole, the economic feasibility. For example, while sugar cane is the main substrate used for bioethanol production in Brazil, most ethanol produced in the United States is from corn.

Bioethanol production from cassava has very high potential in Nigeria as well as in many other African countries because of its ability to give moderate yield even in poor soils. Although cassava yield per hectare is still low in Nigeria (about 11 ton/h), Nigeria has remained the largest cassava producing country in the world (FAO-STAT, 2012). Cassava can be grown on marginal lands where other crops cannot grow. It is therefore suitable for poor rural farmers who cannot afford to buy fertilizers for increased yield. In addition, it can be cultivated two to three times in a year since it does not require so much rain (Hillocks et al., 2002). Thus, we have been working on development of technologies for fuel ethanol production from cassava (Ogbonna and Okoli, 2007, 2009, 2010a, 2010b, 2011). The present study is on a simple process for on-farm fuel ethanol production from cassava and its economic feasibility for commercial on-farm fuel ethanol production in rural communities.

## MATERIALS AND METHODS

### Microorganisms

*Aspergillus awamori* IAM8928 and *Saccharomyces cerevisiae* IR2

(Kuriyama et al., 1985) were obtained from the Department of Microbiology, University of Tokyo, Japan. *A. awamori* was maintained in potato dextrose agar (PDA) while *S. cerevisiae* was maintained in nutrient agar. Each microorganism was maintained in slant cultures and was sub-cultured once every two to three weeks.

### Preparation of cassava flour

Tubers from cassava variety 98/2101 were harvested at the age of 12 months from Ebonyi State Agricultural Development Program (EBADEP), Nigeria. They were peeled, washed and soaked in water over night. The next day, they were removed from water, drained, sliced and sun dried to a moisture content of about 15% in palm basket trays. The trays were taken inside at night and during bad weather. The dry slices were ground into flour using a manual grinding machine and sieved using a muslin cloth.

### Preparation of koji

The cassava *koji* was prepared by solid state cultivation of *A. awamori* using the cassava flour supplemented with 20% rice bran (Ogbonna and Okoli, 2010a). The cassava flour (80 g) was mixed with 20 g of rice bran. The mixture was wetted with 60 ml of water and steamed over a moderate flame for 30 min. They were allowed to cool to room temperature and then inoculated with 5 ml of *A. awamori* spores ( $6.6 \times 10^6$  spores/100 g). It was properly mixed, wrapped in a muslin cloth and incubated at room temperature for 24 h. It was mixed again, incubated for another 24 h and the resulting *koji* was used as enzyme source for cassava flour hydrolysis.

### Preparation of crude enzyme

An airlift bioreactor was used for preparation of crude enzyme (Ogbonna and Okoli, 2010b). The composition of the medium used for enzyme production consisted of (in g/l) cassava starch, 20; polypeptone, 5; yeast extract, 2;  $\text{KH}_2\text{PO}_4$ , 1;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1; and anti-foam agent, 0.1 ml. The medium (3.0 l) was autoclaved and transferred into the sterilized bioreactor. The bioreactor was sterilized by washing very well and filling with 0.1% sodium hypochlorite overnight, followed by rinsing with sterilized distilled water. The reactor containing medium was then inoculated with *A. awamori* spores (initial concentration =  $6.6 \times 10^6$  spores/ml). Aeration was controlled at 0.2 vvm using an air flow meter. After 96 h, the liquid medium was centrifuged at 3000 rpm and the supernatant was decanted into sterile flasks and used as the crude enzyme.

### Production of yeast inoculum

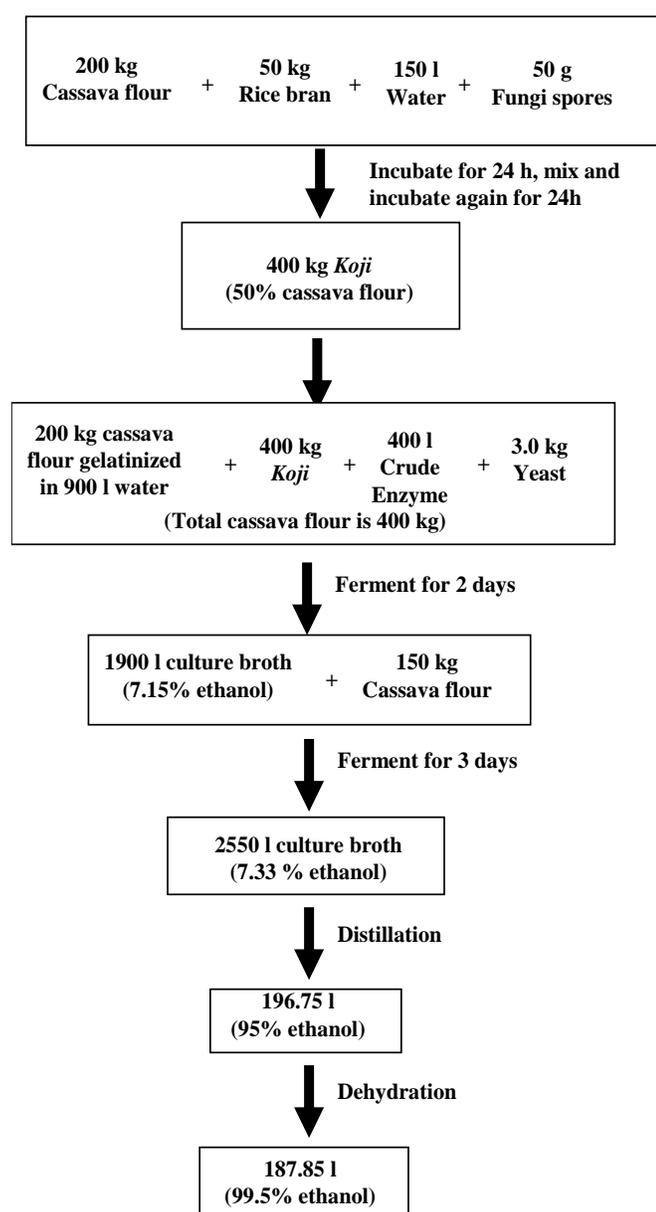
Yeast inoculum was prepared in an airlift bioreactor. The medium used for yeast cultivation was composed of (in g/l) yeast extract, 2.5; polypeptone, 2.5 and glucose, 20. All the media components were dissolved in tap water in a 5000 ml flask and then autoclaved at 121°C for 15 min. The autoclaved medium was transferred into sterilized bioreactor. Sterilization of the bioreactor, and aeration rates were as described for crude enzyme preparation. After the 72 h of cultivation, the yeast cells sedimented at the bottom of the bioreactor and the cells were then harvested by decanting and blotting to remove free water.

### Ethanol production from cassava flour

Based on our previous works (Ogbonna and Okoli, 2010a, 2011),

**Table 1.** Optima conditions for bioethanol production from cassava flour.

Factor	Optima condition
Substrate for <i>koji</i> preparation	80% cassava flour + 20% rice bran
Ratio of water (l) to substrate (kg) for wetting before steaming	60 :100
Spore inoculum concentration	$6.6 \times 10^6$ spores/100 g substrate
Length of <i>koji</i> cultivation	48 hours (with mixing after 24 hours)
Ratio of <i>koji</i> to cassava flour (the cassava flour should be gelatinized in 4.56 volumes of water before mixing)	20 g <i>koji</i> :17.5 g cassava flour
Amount of crude liquid enzyme	1.0 l/kg of <i>koji</i>
Amount of yeast cells	3 g wet weight per 200 g <i>koji</i>
Length of fermentation after yeast inoculation	5 days



**Figure 1.** Schematic flow diagram of a process for production of 187.85 l of ethanol from cassava.

the optimal conditions for ethanol production from cassava, using cassava *koji* and *A. awamori* culture broth (crude enzymes) for hydrolysis are summarized in Table 1. Ethanol production from cassava flour under the optima conditions was done as follows: Cassava *koji* was prepared by mixing 4 kg of cassava flour with 1.0 kg of rice bran and 3.0 l of water. The mixture was steamed and inoculated with  $2.64 \times 10^8$  spores of *A. awamori*. After incubating the *koji* for 48 h, the wet weight was 8.6 kg. In case A, *koji* (4.0 kg), 3.5 kg of cassava flour gelatinized in 16.0 l of water and crude enzyme (4 l) were added into a 50 l fermentation vessel. This was followed by addition of 30 g of yeast paste and 5 capsules of 250 mg chloramphenicol. The total volume of culture broth in the fermentation vessel was about 27.5 l. Starch hydrolysis and ethanol fermentation were allowed to proceed for five days with manual mixing once a day. In another fermentation vessel (case B), 4 kg of *koji* and 2 kg of cassava flour gelatinized in 9 l of water were mixed. Crude liquid enzyme (4 l), 30 g yeast paste and 5 capsules of 250 mg chloramphenicol were added to the vessel. Starch hydrolysis and ethanol fermentation were allowed to proceed for two days. At the end of the 2<sup>nd</sup> day, 1.5 kg of cassava flour was gelatinized in 7 l of water and added to the fermentation vessel. Starch hydrolysis and fermentation were allowed to proceed for another 3 days. Ethanol concentrations in the fermentation vessels were determined on the second, third and fifth day.

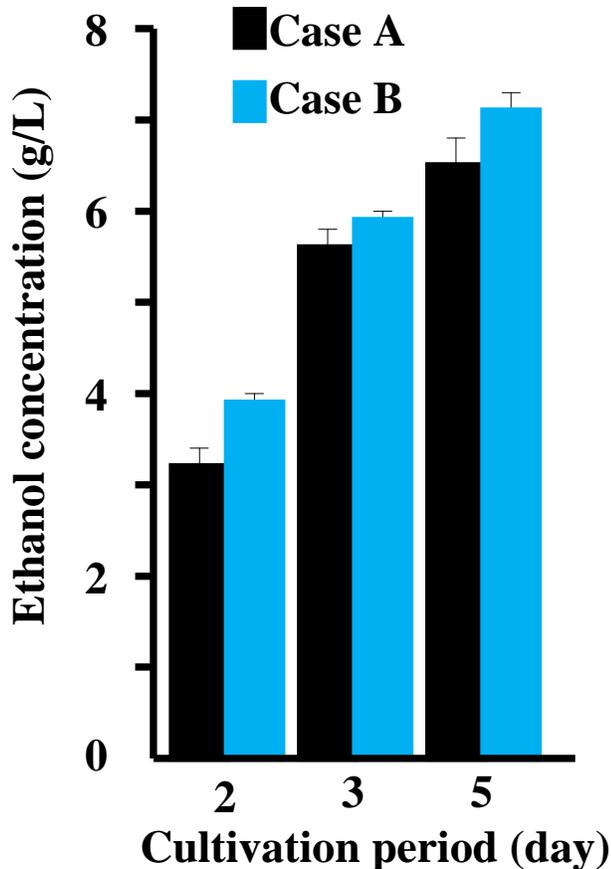
#### Measurement of ethanol concentration

Ethanol concentration in the culture broth was determined as described previously (Ogbonna and Okoli, 2010a).

#### Economic analysis of the developed process

Economic analysis of a process for production of 563.55 l (187.85 l x 3) of 99.5% ethanol per day was made. The developed process was scaled up 100 times with three batches per day and the effects of cost of fresh cassava on the ethanol production costs and relative contributions of other cost components were determined.

The flow chart of the process for production of 563.55 l of 99.5% ethanol per day is shown in Figure 1. *Koji* is prepared by mixing 600 kg of cassava flour with 150 kg of rice bran, 450 l of water and 150 g of *A. awamori* spores. This is incubated in *koji* tray for 24 h, mixed and incubated for another 24 h. The resulting cassava *koji* (about 1200 kg) is divided into three. Each part (400 kg of cassava *koji*) is mixed with 200 kg of cassava flour, gelatinized in 900 L of water. This is followed by addition of 400 l of crude enzyme (or 1.0 kg of commercial amylase suspended in 400 l of water), and 3 kg (fresh weight) of active yeast pre-culture. The total amount of cassava flour in this broth is 400 kg. This is fermented for two days,



**Figure 2.** Effect of mode of cultivation on ethanol production. In case A, 3.50 kg of cassava flour was gelatinized and added to the *koji* and yeast cells but in case B, 2.0 kg of cassava flour was gelatinized and added initially, followed by addition of gelatinized 1.50 kg cassava flour after two days of fermentation.

days, resulting in 1900 l broth with 7.15% ethanol (Estimated from Equation 1). Another 150 kg of cassava flour gelatinized in 500 l of water is added, bringing the total amount of cassava flour to 550 kg. This is fermented for a further 3 days, giving 2550 l of broth with 7.33 % ethanol (Estimated from Equation 1). It is then distilled to yield 196.75 l of 95% (Estimated from Equation 2) ethanol, and dehydration of the ethanol by passing through molecular sieve column will yield 187.85 l of 99.5% ethanol per batch, giving a total of 563.55 l for the three batches.

$$E\% = (Q \times Y \times 100)/V \quad (1)$$

where, E% is the ethanol concentration in the broth; Q is the total quantity of cassava flour used for the cultivation; Y is the ethanol yield from cassava flour; and V is the total volume of the culture broth. The value of ethanol yield (Y) used in the calculation was 0.34 g-ethanol/g-cassava flour, which is the value obtained in this work. The volume of ethanol produced after distillation (Ev) was estimated from Equation 2.

$$E_v = (C_1 \times V)/C_2 \quad (2)$$

Where, C<sub>1</sub>, is the concentration of ethanol in the culture broth before distillation; C<sub>2</sub>, is the concentration of ethanol after distillation; and V is the total volume of the culture broth before distillation.

## RESULTS

### Small scale ethanol production from cassava flour

The results obtained when the 3.50 kg of cassava flour was gelatinized and added at the same time (case A) was compared with the values obtained when 2.0 kg of cassava flour was gelatinized and added initially, followed by addition of gelatinized 1.50 kg cassava flour after two days of fermentation (case B). As shown in Figure 2, the ethanol concentrations on the second, third and fifth day for case A were 3.24, 5.6, and 6.5 %, respectively. These compared with 3.9, 5.9 and 7.05% obtained for case B for day 2, 3 and 5, respectively. The final ethanol concentrations (on the fifth day) correspond to ethanol yields of 0.29 g-ethanol/g-cassava flour and 0.34 g-ethanol/g-cassava flour for case A and case B, respectively. Thus, the method used in case B was adopted.

### Economic analysis of a process for production of 563.55 l of 99.5% ethanol per day

#### Investment costs

The estimated investment costs of a process for on-farm production of 563.55 l of 99.5% ethanol per day are shown in Table 2. *Koji* is produced daily and since each batch lasts for 48 h, two *koji* trays are required to ensure daily production and each is estimated to cost ₦20,000. Three fermentation tanks will be required per day (one for each batch). One production cycle comprise 5 days of fermentation and one day for cleaning and sterilization of the tanks. Thus, the total number of fermentation tanks required for daily production is 18 (3 x 6 days). Each locally fabricated galvanized metal tank is estimated to cost ₦50,000. The distillation system comprises a 1,500 l-pot still, a rectification column and two molecular sieve (zeolite 3a) columns for ethanol dehydration. The pot still, the condenser and molecular sieve columns are locally fabricated while the rectification column and the molecular sieve (zeolite 3a) will be imported. Zeolite 3a now costs between US\$0.7 to US\$1.01/kg. Thus the estimated cost of the distillation system is ₦3,000,000 (US\$18,750). Storage tank for the dehydrated ethanol, cassava milling machine and a tank for gelatinization of cassava flour are also required and the estimated costs are shown in Table 2. A fairly used truck vehicle will be purchased at ₦2,000,000 for transportation of raw materials and finished product. An on-farm open hall building will be constructed at a cost of ₦1,000,000 while an additional ₦150,000 is provided for purchase of minor equipment such as buckets, floor washing equipment and office equipment. The total investment cost is seven million, five hundred thousand naira only (US\$46,875).

#### Operation costs

A breakdown of the operating costs is shown in Table 3.

**Table 2.** Estimated investment costs for ethanol production from cassava flour.

Item	Cost (Naira)
Wooden <i>koji</i> tray (2 at ₦20,000 each)	40,000
Fermentation tanks (made of coated galvanized metal sheets (18 at ₦50,000 each)	900,000
A 1,500 l pot distiller with two molecular sieve columns	3,000,000
Storage tank	100,000
Chip milling machine	100,000
Gelatinization tank	50,000
A truck vehicle	2,000,000
Construction of an open hall building	1,000,000
Other minor equipment	150,000
Total	7,500,000

Currently, US\$1 = N160.

**Table 3.** The operation costs used for economic analysis.

Component	Cost (Naira)
Cassava flour	10,000/ton
Depreciation value	125,000/month
Auxiliary materials	900/ton
Distillation	300/ton
Labour	105,000/month
Utilities	30,000/month
Co-products	-20/Litre

Currently, US\$1 = N160.

The cost of cassava tuber was ₦10,000 per ton. Depreciation value was based on 5 year payback period. Straight line depreciation was used with no salvation and thus calculated as the total investment cost divided by the payback period (the expected life expectancy of the equipment), giving ₦125,000 monthly. If the initial capital is borrowed, the monthly depreciation value will be used to pay back the loan. Interest on loan was not considered since Nigerian Federal Government, through Bank of Agriculture and Bank of Industry provides interest-free or low interest loans (See discussion for details).

The auxiliary materials included the costs of rice bran (30.3 kg/ton), amylase enzyme (1.0 kg), and active dry yeast (1.0 kg). Currently, rice bran is collected free from rice mills in Nigeria, thus a price of ₦380 was budgeted for collection. Technically, it will be easier and even cheaper to purchase enzymes and active yeasts rather than in-house production. Active dry yeast is sold at US\$5.99 per 8.0 kg pack and 1.0 kg (US\$0.749 = ₦120) is sufficient to replace 1.8 kg of wet yeast paste used in this experiment. Furthermore, 1.0 kg of amylase (currently sold at US\$2.5/kg = ₦400) is sufficient to supplement the *koji* enzymes. Thus the total cost of the auxiliary materials is ₦900/ton of cassava tuber. The cost of distillation shown in the table represents the cost of propane

**Table 4.** Composition of 1000 kg of fresh tuber.

Part	Weight (kg)	Percentage
Water	594.2	59.42
Outer skin	10.4	1.04
Inner skin	32.5	3.25
Pulp	337.6	33.76
Chaff	25.3	2.53

gas used for the distillation and dehydration.

A 50 kg of gas (₦6,000) can distill 12 batches (19.8 tons of cassava) and consequently, the cost of distillation is estimated to be ₦300/ton. The cost of utilities (water and electricity) is ₦30,000 per month while the labour cost is ₦105,000/month. This includes four people for peeling of the tubers with monthly salary of ten thousand naira per month each (total of ₦40,000 per month), two secondary school leavers (₦12,000 per month), one driver (₦15,000 per month) and one HND or graduate (₦26,000 per month). The major co-product is the organic manure from the composting of cassava peels and other solid wastes.

As shown in Table 4, about 43 kg of wastes (the outer and inner skins) are produced per ton of fresh tubers. The remnant of the total solid matter in the effluent from distillation pot after separation of the solid distillers wastes is poured into the farm directly as manure. Organic manure is now sold at about one hundred naira per 20 kg (poultry manure is sold at two hundred naira per bag of about 20 kg).

Thus, two hundred naira can easily be realized from manure from one ton of cassava tuber. Another major co-product is the dried distiller's waste (DDW) which is the dried residual solid waste from distillation. It is high in protein, carbohydrate and minerals and used as animal feed.

**Table 5.** Effect of cassava fresh tuber cost on ethanol production costs.

Fresh cassava tuber (N/ton)	Annual costs (x N1000/year)							Total ethanol produced (l) (h)	Unit cost (₦/l) (i)	Value of co-products (₦/l)	Final production cost (₦/l)
	Cassava (a)	Utilities (b)	Auxillary (c)	Distillation (d)	Labour (e)	Depreciation (g)	Total cost				
10,000	11385	360	1024.65	341.55	1260	1500	15871.1	129616.5	122.5	-20	102.5
9,000	10246.5	360	1024.65	341.55	1260	1500	14732.6	129616.5	113.7	-20	93.7
8,000	9108	360	1024.65	341.55	1260	1500	13594.1	129616.5	104.9	-20	84.9
7,000	7969.5	360	1024.65	341.55	1260	1500	12453.6	129616.5	96.1	-20	76.1
6,000	6831	360	1024.65	341.55	1260	1500	11317.1	129616.5	87.31	-20	67.31
5,000	5692.5	360	1024.65	341.55	1260	1500	10178.6	129616.5	78.53	-20	58.53
4,000	4554	360	1024.65	341.55	1260	1500	9040.1	129616.5	69.74	-20	49.74

(a), 550 kg of cassava flour (1650 kg of fresh cassava tubers) is used per batch and with three batches per day, a total of 4.95 tons of fresh cassava is used per day. If we operate for 20 days in a month, 99 tons are used per month, and operating for 11.5 months per year gives 1138.5 tons of fresh cassava tubers per year. The total cost of cassava is thus 1138.5 x cost of cassava tubers per ton (column 1); (b), calculated as ₦30,000 x 12; (c), calculated as ₦900 x 1138.5; (d), Calculated as ₦300 x 1138.5; (e), calculated as ₦105,000 x 12; (f), calculated as ₦15,000 x 12; (g), calculated as ₦125,000 x 12; (h), calculated as 187.85 x 3 x 20 x 11.5 (Figure 1); (i), calculated as total cost divided by the total volume of ethanol.

It is used to replace a part of corn and soybean meals in animal feed. Starch content of the pulp is 75% and the remaining 25% is mainly lignocellulosic materials which are not hydrolyzed during hydrolysis and fermentation. This will amount to 84.4 kg (25% of 337.6) per ton of fresh tuber. This can be mixed with the 25.3 kg of chaff (Table 4) giving a total of 109.4 kg.

The market prices for comparable products (brewers' grains, soybean meal, and palm kernel cake) range from twenty to thirty thousand naira per ton. If a conservative price of fifteen thousand naira per ton is taken, then one ton of fresh cassava will yield DDW worth one thousand six hundred and thirty five naira ((109.4 x 15,000)/1000).

The total amount that can easily be realized from co-products from one ton of cassava is thus one thousand eight hundred and thirty five naira (₦200 + ₦1,635). Since one ton of fresh cassava tubers yields about 90 l of ethanol, manure and DDW will reduce the cost of ethanol by more than twenty naira per liter (₦20/l).

### Effects of cost of fresh cassava tubers on ethanol production costs

The effects of cost of fresh cassava tubers on various cost components are summarized in Table 5. With a price of ₦10,000 per ton of fresh cassava, the production cost is ₦102.5 per liter. Thus, the process is not economical if cassava tubers are purchased at ₦10,000/ton. The cost of production decreases sharply with decrease in the cost of the fresh cassava tubers. If the price of fresh cassava tubers can be reduced to ₦5,000 per ton (for example, by vertical integration of cassava farming and ethanol production), the production cost will be ₦58.53/l. With the current gasoline pump price in Nigeria as ₦97/l, ethanol can be sold at ₦64/l (since the energy content is 66%). Under this condition, the actualized internal rate of return (calculated quarterly for five years by using online IRR calculator ([www.pinegrove.com](http://www.pinegrove.com)), is 17.0255% with a gross return of 47.27%, total return of ₦11,045,020 and net cash return of ₦3,545,020.

### Effect of cost of fresh cassava tuber on the relative contributions of various cost components

As shown in Table 6, as the cost of cassava tuber decreases, the relative contributions of auxiliary costs, distillation and labour costs increase. The cost of fresh cassava tubers has an overwhelming effect on the final ethanol production costs.

The final ethanol production cost decreases linearly with decrease in the cost of fresh cassava tubers (Table 5). The cost of cassava makes up between 50.38 and 71.73% of the final production cost.

The relative contribution of other cost components increase with decrease in the cost of cassava tubers.

The auxiliary and labour costs increase up to 11.33 and 13.94%, respectively, when the cost of cassava tubers decrease to ₦4,000 per ton. The relative contribution of distillation cost remains very low regardless of the cost of fresh cassava tubers.

**Table 6.** Effects of cassava cost on relative contributions of various components on ethanol production costs.

Cost of fresh cassava tubers (₦/ton)	Contribution to overall production costs (%) cost components			
	Auxiliary	Distillation	Labour	Cassava
10,000	6.46	2.15	7.94	71.73
9,000	6.95	2.32	8.55	69.55
8,000	7.54	2.51	9.27	67.0
7,000	8.23	2.74	10.12	63.98
6,000	9.05	3.02	11.13	60.36
5,000	10.07	3.36	12.39	55.93
4,000	11.33	3.78	13.94	50.38

Other components such as utilities, and depreciation together make up 11.72 to 20.57% of the total production cost, depending on the cost of fresh cassava tubers. These values were calculated as the values for each component in Table 5 divided by the total production cost at each cassava tuber price.

## DISCUSSION

The results of ethanol production from cassava flour, using cassava *koji* supplemented with crude liquid enzyme showed that instead of adding 3.5 kg of gelatinized cassava flour at the start of the experiment, adding 2 kg initially and then the remaining 1.50 kg after two days (fed-batch culture) gave higher ethanol concentration and yield. When the whole 3.50 kg was added initially, the viscosity of the broth was very high and this would reduce mass transfer and thus ethanol productivity. The process for fuel ethanol production from cassava flour shown in Figure 1 is simple, and can be operated even in rural communities without electricity supply.

The above economic analysis has shown that at the current market fresh cassava tuber cost of ₦10,000, the cost of cassava accounts for 71.73% of ethanol production costs. Other workers have also noted that the cost of raw materials accounts for 70 to 76% of ethanol production (Krishna et al. 2000; Zhang et al., 2003, Li and Chan-Halbrendt, 2009). Even though Nigeria has remained the world largest producer of cassava for many years now (FAO-STAT, 2012), the cost of cassava tubers in Nigeria (ten thousand naira per ton = US\$62.5) is much higher than the US\$24.1 to US\$27.71/ton reported for China (Zhang et al. 2003) and the current international prices of US\$36 to 40/ton (₦5,760 - 6,400).

The process described in this paper is not economically viable if cassava tubers are to be purchased from the open market at the current prices. However, there is a sharp seasonal and annual fluctuation in the costs of cassava tubers in Nigeria. Generally, the cost during the rainy season can be less than half of the price during the dry season. It has also been reported that with new cassava varieties, the total cost of production of cassava tubers ranges from ₦3701.56/ton to 4,555.769/ton, depending on farm location within the South-South and South-East parts of Nigeria (PIND, 2011). Furthermore, the farm gate price is usually much lower than the market

prices and in some rural areas, cassava tubers are sold for as low as ₦3000/ton, especially during the rainy seasons (April to October). On-farm ethanol production in a vertically integrated system, where the investor also operates a cassava farm, is therefore recommended. In such a case, the cost of cassava will be much cheaper and the influence of cassava tuber market fluctuation on profitability can be avoided. The cost of transportation of the finished products to petrol stations or central blending facility has not been included. The company truck will be used for the transport and the small quantity (5,610 l per month) can easily be sold within the locality. The additional costs due to transportation will depend on the transportation distance. Furthermore, interest on loan was not included in this economic analysis. However, in some cases, loans from the Bank of Agriculture or Bank of Industry attract 2% interest (cost of borrowing, risks and expenses). Furthermore, the interest rate on loans from commercial banks in Nigeria is currently about 20%. Thus, depending on whether a 2% interest loan is obtained from Bank of Industry/Bank of Agriculture or 20% interest loan is obtained from a commercial bank, the cost of production will increase by either ₦1.157/l or ₦11.57/l respectively.

The production costs of ₦58.53/l at ₦5,000/ton is lower than the €0.38 0.54/l for ethanol production from starchy crops but higher than the production cost from sugar cane in Brazil (€0.23-0.28/l) (Budimir et al., 2011). It also compares with US\$0.235-0.365/l for ethanol production from dry corn mills in United States (Kwiatkowski et al., 2006). Generally, the production cost depends on the raw material and the country (because of differences in the labour, utility and fixed costs) and ranges from US\$0.211/l for sugar cane in Brazil to US\$0.762/l for sugar beet in EU (Li and Chan-Halbrendt, 2009). The international price of fuel ethanol is currently between US\$0.596/l and 0.747/l. Thus, local production, even at the current fresh tuber price of ₦10,000/ton, is better than importation and will also provide employments (both in the farm and ethanol production plants) and thus help in

socio-economic development of the rural communities. Currently, Nigeria and many countries provide subsidies on fuels. Thus, fuel ethanol production will even be more profitable if such subsidies are extended to fuel ethanol.

The above results have shown that African nations must make effort to improve agricultural productivity if they are to benefit from the huge potentials of bio-energy production. The yields of most crops in Africa is less than 30% of the yields in the United States and other developed countries (FAO-STAT, 2009) and Governments should encourage both local and foreign investors in Agriculture as a means of improving productivity, reducing prices, and improving food security level. African nations must emulate many developed and even developing countries who support Agriculture and Bioenergy industries by providing subsidies and various forms of incentives.

One argument against the use of food crops for fuel ethanol production is that it can adversely affect food security. It is very important to point out that food security cannot be discussed without energy security. Food production and distribution are highly depended on energy supply. In Nigeria, for example, increase in fuel prices often results in sharp increases in the prices of all other items including all the food items. World bank (2013) report showed that there are more than 202 million hectares of usable uncultivated land in Africa and blamed land governance on the inefficiency on land use in Africa. Gnansounou et al. (2007) reported that in some countries only about 6% of arable land is under effective cultivation. Thus, with proper planning and land use management, energy crops can be produced on large scales without putting constraint on land availability for food crop production.

In conclusion, the simple process described in this paper for fuel ethanol production from cassava can be economically viable and feasible for rural communities in many African countries provided that the investor(s) also establish cassava farms and that the production is sited within the farm to ensure steady supply of cassava at less than ₦5,000/ton. Commercial bioethanol production in rural communities will reduce the present high unemployment rates, thereby leading to socio-economic development of the rural communities.

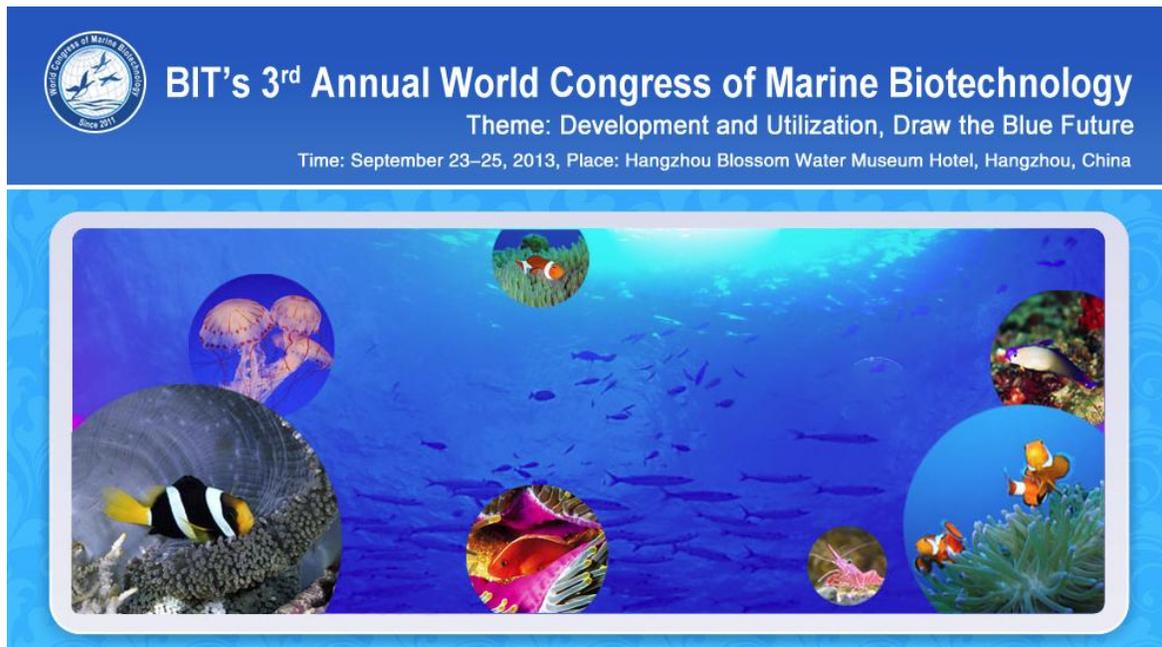
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### BIT's 3rd Annual World Congress of Marine Biotechnology, Hangzhou, China, 23 Sep 2013



The banner features a blue background with a white border. On the left is the logo for the World Congress of Marine Biotechnology, which includes a circular emblem with a fish and the text 'World Congress of Marine Biotechnology Since 2011'. To the right of the logo, the text reads: 'BIT's 3rd Annual World Congress of Marine Biotechnology', 'Theme: Development and Utilization, Draw the Blue Future', and 'Time: September 23-25, 2013, Place: Hangzhou Blossom Water Museum Hotel, Hangzhou, China'. Below the text is a large rectangular image showing a vibrant underwater scene with various marine life, including jellyfish, clownfish, and a large shell, all set against a blue background with light rays filtering down.

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