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ACTIVATION OF OXIDOREDUCTASES IN MILLET AND COWPEA GRAINS IMPROVES PROTEIN UTILIZATION FOR GROWTH

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ACTIVATION OF OXIDOREDUCTASES IN MILLET AND COWPEA GRAINS IMPROVES PROTEIN UTILIZATION FOR GROWTH.

By

Lovisa Hinandyooteti Kambonde

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ABSTRACT

ACTIVATION OF OXIDOREDUCTASES IN MILLET AND COWPEA GRAINS IMPROVES PROTEIN UTILIZATION FOR GROWTH.

By

Lovisa Hinandyooteti Kambonde

Cowpea (*Vigna unquiculata* L.) is a legume that provides an affordable source of protein for many regions in Africa where animal sources of protein are not affordable. Cowpea is especially important for providing protein for weaning foods. Millet (*Panicum miliaceum L.)* is used in Africa, India and in some parts of Asia as a source of energy. During germination, oxidoreductases reduce disulfide bonds of less digestible proteins in grains. Germination has also been used to decrease the anti-nutrient content, enhance consumption, and in some cases, increase the bioavailability of proteins. The aim of this study was to evaluate protein quality of germinated and extruded millet and cowpea foods. It was hypothesized that germination will increase the bioavailability of cysteine, particularly in cowpea, thus increasing growth potential. Millet and cowpea were processed by extrusion and conventional cooking and evaluated for growth potential and protein digestibility. The study showed that a blend of millet plus cowpea diet yielded the greatest growth in weanling rats. Germination did not affect protein digestion, however germination yielded higher food intake (37.9g) in comparison to non-germinated feed (35.2g). Extrusion processing retained higher protein digestibility of millet than cooking. Germinated feeds also resulted in more growth (51.1g) than non-germinated feeds (43.1g), strongly suggesting that germination increased the bioavailability of cysteine.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

ACRONYMS

AAS	- Amino Acid Score
BV	- Biological Value
FAO	- Food and Agriculture Organization of the United Nations
FAOSTAT	- Food and Agriculture Organization Statistic Database
IITA	- International Institute of Tropical Agriculture
NDB	- Nutrient Database
NPU	- Net Protein Utilization
PDCAAS	- Protein Digestibility Corrected Amino Acid Scores
PER	- Protein Efficiency Ratio
RVU	- Rapid Visco Units
TPD	- True Protein Digestibility
USDA	- United State Department of Agriculture
WHO	- World Health Organization

I INTRODUCTION

Cowpea is one of the important legume crops consumed in many parts of the world. Because of its high protein content, this crop has a potential to influence the nutritional profile of foods in countries where animal protein is unaffordable and/or not readily available. However, the potential benefits can be limited by the presence of anti-nutritional factors such as hemagglutinins (Oyeleke et al, 1985). Millet has been used for centuries in India, parts of Africa and Asia as a staple food to provide energy. Millet is hardy and grows in hot and dry areas, adapting to local environments. Millet is more efficient in utilization of soil moisture, has a higher level of heat tolerance and grows better in these conditions than sorghum and maize ((FAO, 1991).

Cowpea is relatively high in protein (20-35%) and has therefore been used to improve weaning foods in developing countries (Jirapa et al, 2001; Oyeleke et al, 1985). Methods have been developed (e.g. germination) to increase the bioavailability of legume and cereal nutrients. During germination, a complex metabolic process takes places, where lipids, carbohydrates and storage proteins in the seeds are broken down to energy and amino acids (Urbano et al, 2005). This improvement is usually as a result of breakdown of complex carbohydrates and protein into smaller and more digestible molecules. Oxidoreductases are enzymes that participate in redox reactions during germination, and they reduce the disulfide bonds of storage proteins and proteins in the starchy endosperm. This reduction leads to the increased susceptibility of proteins to proteolysis. Oxidoreductases also cause a reduction of anti-nutrients by inactivating specific enzyme inhibitors (e.g. protease and amylase, Wong et al, 2004).

The preliminary work of the research focused on determining the optimum time for germination and inactivation of phytohemaglutinin activities in both millet and cowpea. The main focus of the research concentrated on evaluating the effect of germination, processing methods (extrusion and traditional cooking) on protein digestibility of millet and cowpea.

Millet and cowpea were allowed to either germinate or not, and extruded or cooked to produce ingredients for an in vivo study. These products were formulated into 12 diets. The 12 diet formulations were fed to male weanling rats to evaluate protein digestibility, food intake and growth potential. Therefore the **overall objective** of these studies was to investigate the effect of germination and processing methods on protein utilization of millet and cowpea.

Thus the following hypothesis were tested:

1. Germination reduces disulfide bonds in some proteins to increase the "soluble protein" fraction and, therefore, growth.
2. Protein utilization is equivalent in extruded and traditionally cooked millet and cowpea.

II LITERATURE REVIEW

2.1 MILLET (*Panicum miliaceum L.*)

2.1.1 Global importance of millet

Millet is one of the oldest foods known to humans and it has been used in Africa and India as a staple food for thousands of years. It is a summer cereal grass with large stems, leaves and heads. Millet is an important food and forage cereal because it is a drought tolerant crop that can be grown on poor, sandy soils in dry areas. It is more efficient in its utilization of moisture than sorghum or maize. Millet grows well on poorly fertilized and dry soils and fits well in hot climates with short rainfall periods and cool climates with brief warm summers. The plants need good drainage, have a low moisture requirement and do not grow well in waterlogged soils. Millet is unique due to its short growing season. It can develop from a planted seed to a mature, ready to harvest plant in as little as 65 days (FAO,1991).

2.1.2 Uses of Millet

In Africa, millet is used to make bread, baby food, thick or stiff porridge and ontaku/oshikundu, (a traditional non alcoholic beverage). It is also used as a stuffing ingredient for cabbage rolls in some countries. Millet is a gluten free grain and lack of gluten makes it a safe food for individuals who suffer from wheat allergies. Millet can be used in pilafs, casseroles or most oriental dishes.

2.1.3 Millet porridge – common in Africa

Millet dishes vary according to the customs, culture and cultivars of each region. Millet flour is used to make thin porridge, made by adding 10% flour to 90% water and boiling for 10 to 15 minutes. This product is mainly consumed by children, because it has thin consistency. Adults, consume thin porridges made from millet at breakfast, with fresh or sour milk, butter, groundnut flour, sugar, honey, or limejuice as additives (FAO, 1991). Thick porridges, soured or not, are made by adding approximately 40% (w:v) flour into boiling water and stirring until the desired consistency is attained. The porridge is then cooled and eaten with meat, vegetables, legumes or sour milk to enhance its palatability as well as its nutritional value.

Germination of small grains has been shown to have beneficial effects in reducing the bulk density of food (FAO, 1991) and thereby increasing its energy density. Souring the porridge by the addition of a small quantity of starter that has been fermented for up to 48 hours is widely reported for Botswana, Tanzania, Zimbabwe, Zambia and Swaziland (FAO, 1991). Soured porridges can be either of thin or thick consistency.

Thick porridges are a staple food in many African countries. Most meals feature stiff porridges as a central element. According to the United Nations Food and Agriculture Organization, "Cereals account for as much as 77% of total caloric consumption in African countries, and contribute substantially to dietary protein intake (FAO, 1995). The majority of traditional cereal-based foods consumed in Africa are processed by natural fermentation.

Millet thick/stiff porridge is called *oshithima*, in Namibia, garri in parts of West Africa and *ugali* in East Africa. In Africa, porridges are generally served thick, with a solid consistency that can be shaped and eaten with fingers, and are often served with saucy stews and vegetables. It can also be served with fermented milk.

2.1.4 Millet varieties

There are many varieties of millet, but the four major types are Pearl, which comprises 40% of the world production, Foxtail, Proso, and Finger Millet. Pearl Millet produces the largest seeds and is the variety most commonly used for human consumption. Pearl millet is the most widely grown of all millets. It is a traditional crop grown in Western Africa, particularly in the Sahel, however it is also grown in Central, Eastern and Southern Africa. It is also known as bulrush millet, babala, bajra, cumbu, dukhn, gero, sajje, sanio or souna (common names given in different countries).

Proso millet has been recently introduced as a grain crop in the South Eastern coastal plain of the United States, and is grown in Colorado, North Dakota, and Nebraska. Most of the crop is used for livestock, poultry, and bird feed with very little use for human consumption. It is planted in spring and harvested in the fall and is often grown in rotation to wheat, using the same planting and harvesting equipment (USDA, 2003). Finger millet was once widely grown in Southern Africa, but its uses have been overtaken by pearl millet. Finger millet is currently used as principle cereal grain in Uganda. It is also used in India as popped grain and in Ethiopia to make arake, a strong distilled liquor (Van Wyk & Gericke, 2000). Foxtail millet is one of the oldest cultivated

crops. It was used in India, China and Egypt before written records existed. This type of millet is still used in Eastern Europe for porridge and bread and for making alcoholic beverages (FAO, 1996).

2.1.5 Description of Millet plant and seed.

Figure 2.1: Structure of a typical millet plant with seeds growing on condensed panicles. Source: www.fao.org/docrep/w10808e/w1808e00.htm

Millet is a tall erect annual grass with an appearance strikingly similar to maize. The plants will vary in appearance and size, depending on variety, and can grow anywhere from one to 3 m tall. Generally the plants have coarse stems, growing in dense clumps and the leaves are grass-like, numerous and slender, measuring about 2.54 cm wide and up to 1.8 m long. The seeds are enclosed in colored hulls, with color depending on the variety (FAO, 1996). The seed heads themselves are held above the grassy plant on a

spike like panicle 15 to 35 cm long. Because of a remarkably hard, indigestible hull, this grain is generally de-hulled before it is used for human consumption. De-hulling does not markedly lower the nutrient value, as the germ stays intact through the de-hulling process. The de-hulled millet grains look like small yellow spheres with a hilum on one side where it was attached to the stem. This gives the seeds an appearance similar to tiny, pale yellow beads (FAO, 1996).

2.1.6 Nutritional profile of Millet

The nutritional quality of millet grain is directly related to its chemical composition. As a cereal grain, millet grain is an important source of energy in the form of starch, but it can also contribute a significant amount of fiber, mineral and other nutrients to the diet.

The limiting amino acid is lysine, although under better cultivation conditions, millet may contain higher concentrations of lysine and other essential amino acids than sorghum, maize, wheat or rice (Akingbala et al, 2002). It is also a good source of B-complex vitamins (niacin, thiamin, and riboflavin), lecithin, and vitamin E. It is particularly high in the minerals iron, magnesium, phosphorous, and potassium. Protein quality is higher in the outer layers of the endosperm and in the germ, because that is where the lysine-rich albumins (water soluble proteins) and globulins (salt soluble proteins) are located. The lysine poor prolamins (alcohol-soluble proteins) and the glutenins (acid or base – soluble proteins) are located mainly in the inner endosperm of the seeds. In general as the protein content of the cereal increases, its protein quality and digestibility decrease, because the protein increase is mainly the lysine–poor, less digestible, prolamin protein fraction (Kulp and Ponte, 2000).

2.1.7 Goiterogenic substances in millet

Millet has an interesting characteristic in the hulls and seeds contain small amounts of goiterogenic substances (C – Glucosylflavones and their metabolites) that limit uptake of iodine by the thyroid (McDonough et al, 2000). Cooking destroys the enzyme that converts thiocynates into active thyroid function inhibitors, and as such, cooked millet should not be goiterogenic. However, some researchers feel that these "thyroid function inhibitors" cause goiter (Akingbala et al, 2002) and they suggest that the correlation between millet consumption and goiter incidence in some of the developing countries where millet constitutes a significant part of the diet is due to the goiterogenic substances in millet. But, in many of the developing countries where millet is consumed6, another contributing factor may be a lack of sufficient dietary iodine.

2.2 COWPEA (*Vigna unguiculata*)

2.2.1 Global importance of cowpea

Cowpea (*Vigna unguiculata*), an annual legume, is also commonly referred to as southern pea, black eye pea, crowder pea, lubia, niebe, coupe or frijole. This crop originated in West Africa where it was closely associated with the cultivation of sorghum and pearl millet. It is widely grown in Africa, Latin America, and Southeast Asia. In the United State the black-eyed cowpea type is grown primarily in California and is marketed as California black-eyed peas (Davis et al, 1991).

Cowpea plant can be used at all stages of growth as a vegetable crop. The tender green leaves are a significant food source in Africa and are prepared in a same way as spinach. Immature snapped pods are used in the same way as snapbeans, often being mixed with other foods. Green cowpea seeds may be boiled as a fresh vegetable, canned or frozen. Dry mature seeds are canned or eaten after boiling (Davis et al, 1991).

Cowpea seed can provide valuable nutrient component in the human diet. It is rich in protein (18 – 35 %) and carbohydrates (55- 63%). The protein in cowpea seed is richer in lysine and tryptophan compared to cereal grains. Therefore, cowpea seeds are often eaten with cereals to improve the overall protein quantity and quality of the diet. Because of the complex carbohydrates and dietary fiber, cowpea has a relatively low Glycemic Index (G.I.) and is a good source of fiber (Helland et al, 2002). However, due to the long cooking time and presence of the anti nutritional factors, its consumption has been reduced (Leucona – Villanueva et al, 2006). The cowpea seed contain indigestible sugars such as starchyose, raffinose and verbascose, which may cause flatulence in some

individuals, and as such make it an unpopular part of the human diet. Taste is also one of the factors that limit the consumption of cowpea.

2.2.2 Description of cowpea plant and seed

The history of cowpea dates to ancient West Africa cereal farming, about 5 thousand years ago and it was closely associated to sorghum and pearl millet. Cowpea is an annual warm season crop and it adapts very well to many areas of the humid tropics and temperate zones. It can withstand heat and dry conditions, but is intolerant of frost. Cowpea plants are usually as erect and bushy, but other types are "climbers". These growth habits lead to differences from one species to another (Davis et al, 1991). The cowpea seed can develop from a planted seed to a mature, ready to harvest plant in about 90 days. The seed coat can be smooth or wrinkled and of various colors including white, cream, green, buff, red, brown, and black. Many are also referred to as blackeye or pinkeye where the white colored hilum is bordered by another color.

Figure 2.2: Diagram illustrating the primary structure of the cowpea plant.

Source: International Institute of Tropical Agriculture (IITA)

2.2.3 Cowpea varieties

Cowpea plants are usually classified according to their seed type and coat color. Black eye and purple eye—The immature pods shell easily, because the hull is easily bent and the seeds come out of the pod clean and free. The shelled peas are suitable for processing. The white hilum is surrounded by black, pink, or light-red color.

Brown eye—Pods vary in color from green to lavender and have a wide range of lengths. The immature seeds, turn to a medium dark color upon cooking and they became very tender with less natural flavor. Crowder—Seeds are closely packed in the pods and have a globular shape. Cream—Seeds have a cream color and have no noticeable hilum. White acre—This type is kidney shaped with a rounded end and is a semi-crowder, generally tan in color and somewhat small with a hard pod. Each type produces a seed with a distinctive appearance and flavor (IITA, 2006).

2.2.4 Utilization

The dried black eye or purple eye cowpea types are mainly used for food products. The dried beans are commonly sold directly to the consumer after cleaning and bagging (common practice in Africa). Another common use is the canned product. Cowpea is also incorporated in various soups and bean mixes.

2.2.5 Growing practices – African Perspective

Traditionally, cowpea is grown on small farms, usually intercropped with cereals such as millet and sorghum. The use of pesticides is not common because they are expensive and often not available. The cowpea and cereal are usually planted in alternating rows. Millet or sorghum is planted before the cowpea. The fast growth and spreading habit of traditional cowpea varieties suppress weeds, and soil nitrogen is increased which improves cereal growth. The two crops are harvested at different times, making food available for a long period. West Africa is one of the biggest cowpea-growing regions in the world with Nigeria producing 2,317,000 Mt in 2005 (FAOSTAT, 2006). Cowpea is particularly important in West Africa because of its adaptability to the tropical environment.

2.3 ANTINUTRITIONAL FACTORS IN MILLET AND COWPEA

Cereal and legume grains contain compounds called anti-nutrients that reduce the availability of nutrients. The anti-nutritional factors that are common to both millet and cowpea are inhibitors of digestive enzymes (trypsin, chymotrypsin and amylase inhibitors), phenolic compounds, tannins and phytic acids. Lectins are predominantly in legumes (e.g. cowpea).

2.3.1 Protease inhibitor

Proteases inhibitors (trypsin and chymotrypsin inhibitors) bind with trypsin and chymotrypsin and prevent protein hydrolysis. Amylase inhibitors will bind to amylase and prevent starch hydrolysis. Consumption of these inhibitors will cause indigested proteins and starch to pass to the colon where bacteria will use the protein and starch to grow and will produce short chain fatty acids and gas as by-products. Extensive microbial action will cause abdominal swelling (bloating), flatulence, and diarrhea.

If these inhibitors are consumed habitually, the pancreas will secrete more trypsin, chymotrypsin and amylase to compensate or replace enzymes that are bound by the inhibitors. Digestion will return to normal and abnormal discomfort will disappear. Growth depressed by the trypsin/chymotrypsin inhibitor is due to poor digestibility of dietary and endogenous proteins loss of amino acids in the form of enzyme being secreted by hyperactive pancreas. Since pancreatic enzyme are rich in sulfur containing amino acid, the effect of a hyperactive pancreas is to divert the amino acids from the

synthesis of the body tissue proteins to the synthesis of these enzyme that are subsequently lost in the feces (Lajolo F M and Genovese M I, 2002).

2.3.2 Phytohemagglutinin activity

Consumption of lectins is more serious. Lectins bind to glycoproteins on cells that line the digestive tract. As the lectins binds to the glycoprotein on cell membranes, the membrane brakes and the cell dies. Destruction of cells lining the villi of the small intestine causes sloughing of the villi tips, mal-absorption and bleeding. Large, acute doses of lectins result in vomiting, abdominal pain, diarrhea and dehydration, may require hospitalization.

Chronic consumption, such as forcing animals to eat lectins in raw legumes, will result in weight loss, gastro intestinal bleeding and death (Bau et al, 1997). Since lectins bind to glycoproteins of the red blood cells causing clumping of the cells, lectins are also known as hemagglutinis. This property of causing red blood cell clumping is the basis for detecting and quantification of active lectins in foods.

Other nutritional and physiological effects of phytohemagglutinins are lowering insulin levels in blood, inhibition of disaccharides and proteases in the intestines, degenerative changes in the liver and kidneys and intereference with absorption of non- heme iron and lipid from the diet (Linier, 1994).

Lectins, protease inhibitors and amylase inhibitors are heat labile (Owen,1996). Therefore, cooked or roasted foods are safe to consume as long as the foods receive sufficient heat to inactivate these inhibitors. Other common food processing methods such as, steaming, microwave cooking, drum processing and extrusion are efficient in inactivating most or even all of the enzymes inhibitors. The inactivation is dependent on the cooking time, temperature, particle size, and moisture conditions.

2.3.3 Polyphenols, tannins and phytic acids

Polyphenols, condensed tannins and phytic acids all interact with proteins to form insoluble complexes. The resultant complexes reduce access of trypsin and chymotrypsin to peptide bonds, which reduces protein digestibility. Since most of the phenols and condensed tannins are in the hulls, de-hulling millet grain will increase protein digestibility (McDonough et al, 2000).

The brown red and black colors in the hulls of the millet and cowpea are mostly due to the phenolic compounds and condensed tannins. Therefore dark colored varieties have lower protein digestibility than light colored varieties unless hulls are removed.

2.4 GERMINATION

Germination technology is among the simple, inexpensive, easy to carry out and easily adaptable technologies (Ariahu et al, 1999). Germination activates thioredoxins and similar oxidoreductases, which reduce disulfide to produce sulfhydryl form. One purpose of the oxidoreductases during germination is the inactivation of several inhibitors such as amylase, trypsin, and chymotrypsin inhibitors (Wong et al, 2004). When the inhibitors are inactivated, hydrolysis of protein and starch components occurs to provide seedlings with raw materials to grow.

Germination is a simple technology that is sometimes used to hydrolyze starch and reduce bulkiness of cereal foods to stimulate greater consumption of energy (Mbithi-Mwikya et al, 2002). This is very important to infants and small children existing on predominantly cereal diets. Germination may also, decrease cooking time, improve sensory properties, increase vitamin C and of certain B- group vitamin content (Helland et al, 2002) and decrease phytates (Trugo et al, 2000) and reduce the amounts of oligosaccharides. However, germination is also a very active and complex metabolic process that may alter the chemical, structural, and organoleptic properties of the seed, and potentially decrease its nutritive value (Urbano et al, 2005). Reduction of disulfide bonds has the general effect of increasing the solubility of proteins in aqueous solution. Increased protein solubility may enhance digestibility of proteins and specifically increase the bioavailability of cysteine - a sulfur containing amino acid. Sulfur containing amino acids are often the first or second most limiting amino acids in human diets that contain predominantly cereals and legumes.

2.5 EXTRUSION

2.5.1 Extrusion cooking

Transformations that occur during processing is one of the most important factors that distinguishes one food process and food type from another. Extrusion cooking, involves heating to high temperatures for a short time, the application of mechanical mixing and shear force, and formation of a structured product. Extrudates expand during the high temperature, short time extrusion because the dough moisture cannot escape due to high pressure, but instead flashes off as steam at the die exit (Onyango et al, 2004a).

The use of high temperatures and pressure reduce the processing time and allows a full conversion of raw material to its functional form in periods as little as 30 to 120 seconds. It is a relatively low moisture process compared with the conventional cooking (Guy R, 2001). Extrusion process produces a wide variety of foods and ingredients. Some characteristics of extrusion are partial gelatinization of starch and denaturation of protein. However, the degree of protein transformation is influenced by the pre and post processing operations, and their interactions. Other characteristic includes inactivation of many native enzymes and anti nutritional factors, reduction of microbial count, and improvement in digestibility and biological value of proteins (Alarcon – Valdez et al, 2005). The complex physio – chemical changes that occur in carbohydrate rich foods during high temperature short time extrusion are influenced by the operating conditions of the extruder and the rheological property of the food. Extrusion thermo-mechanically denatures and re-orients proteins in starchy foods resulting to changes in digestibility and bioavailability of amino acids (Onyango et al, 2004b).

2.5.2 Extrudate's Starch Viscosity

Unmodified starch granules are generally insoluble in water below 50°C. When Starch granules are dispersed in water above 50°C, the granules absorb large amounts of water and can swell to many times their original size. Above a critical temperature, the starch granules undergo an irreversible process known as gelatinization. Starch begins to gelatinize between 60 and 70°C, the exact temperature is dependent on the specific starch. For example, different starches display different granular densities, which affect the ease with which these granules can absorb water (Onyango, 2004b). The large granules, which are usually less packed together, begin to swell first. The gelatinization range is the temperature ranges over which all the granules are fully swollen. This range is different for different starches. However, gelatinization can be observed visually, because of increased translucency and viscosity. This is due to water being absorbed away from the liquid phase into the starch granule (Guy, 2001).

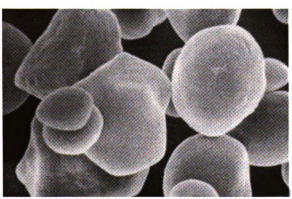


Figure 2.3: Photomicrograph of raw starch with no moisture.

Source: Food Resource Nutrition and Food Management, Oregon State University

2.6 HUMAN REQUIREMENT FOR PROTEIN AND AMINO ACIDS

Proteins are made up of essential amino acids and non – essential amino acids. The former cannot be synthesized in the body, therefore this type of amino acids must be included in the diet and the latter group can be synthesized within the body and therefore do not absolutely need to be in the diet. The essential amino acids are classified as such because of the specific physiological roles they play in promoting growth in children, preventing diseases and maintaining a positive nitrogen balance in children and adults. The essential amino acid that worth mentioning is cysteine that is useful for pre-term infants with metabolic disorder and malnourished patients suffering from compromised liver functions such as cirrhosis. Proteins in the body are continually breaking down and re-synthesized. A large proportion of the amino acids released during tissue protein breakdown are reutilized for the synthesis of new proteins. Amino acids that are not reutilized and those that are consumed in excess of the amounts needed for tissue synthesis are degraded completely and their nitrogen is incorporated into urea, and excreted quantitatively in the urine (Harper and Yoshimura, 1993).

Amino acids need to be consumed to replace the amino acids catabolized by the body. For infant to children below 18, there is a need to consume more protein per kg of body weight because new protein is slowly being deposited in the body (e.g., growth is occurring). Synthesis of new protein requires a greater proportion of essential amino acids and therefore children require better quality proteins than adults.

2.7 PROTEIN QUALITY

Apart from a favorable essential amino acid profile, easy digestibility is an important attribute of a good quality protein. Chemical score does not take into account the digestibility of protein or availability of amino acid. Biological methods based on growth measurement and nitrogen retention assess the overall quality of the protein. These methods include determination of protein efficiency ratio (PER), net protein utilization (NPU), biological value (BV), and true protein digestibility (TPD) (FAO. 1995).

Protein digestibility corrected amino acid score (PDCAAS) is currently the most appropriate method and its use is recommended by FAO. PDCAAS estimates protein nutritional quality by combining information from calculations that determine the most limiting amino acid in a given food protein based on the reference protein and an *in vivo* assay that measures the true protein digestibility of the food protein. The PDCAAS is a better estimate of protein quality for humans than PER that uses rat growth data. Rat growth does not compare very well with the synthesis of protein in human adults, but is more comparable to the human infant growth (Nielsen, 2003).

However protein digestibility by rats, is comparable to digestion of proteins by humans, therefore the PDCAAS method most often estimates true digestibility of protein as determined by the rats. One of the limitations of PDCAAS is that it uses the amino acid score of only one amino acid and ignores the other essential amino acids.

The poor protein digestibility of cereals and legumes is caused by the polyphenols that bind to the enzyme in the digestive tract and thus inhibiting the utilization of proteins and carbohydrates (Onyango et al, 2004 a).

The reaction of the phenolic compounds with the sulfhydryl and amino groups of protein also results in decreased digestibility and availability of protein bound lysine and cysteine (Owen, 1996). Other factors such as the interaction of proteins with the polysaccharides and the interaction between proteins and dietary fiber also reduces the rate and the completeness of protein hydrolysis.

2.8 RATIONALE OF THE RESEARCH

Germination is known to activate thioredoxin and other similar oxidoreductases that in turn enhance protein in aqueous solutions. Preliminary work demonstrated that aqueous soluble proteins were increased when millet and cowpea were germinated for 48 hours. Existing literature suggest that germination has no effect or it slightly increases protein digestibility perhaps by reducing di-sulfide bonds to the sulfhydryl form. The protein digestibility data, however, are derived primarily from *in vitro* studies rather than *in vivo* studies. Based on these findings, further research was designed to determine if increased protein solubility would result in greater *in vivo* protein digestibility. It was rationalized that even if overall protein digestibility was not increased, the bioavailability of cysteine could be increased as a result of germination. Moreover, open kettle cooking or boiling of millet, the usual method to prepare millet-based foods in Africa significantly reduces protein digestibility (Kurien et al, 1961). It has been observed that boiling or open kettle cooking sorghum also reduces the protein digestibility but extrusion retained high degree of sorghum protein digestibility (Mosha T C E, 2004). Since most of the previous studies that measured protein digestibility following germination was done *in vitro*, the effect of germination on millet and cowpea protein digestibility is not well known. Also no one has specifically examined if germination enhances bioavailability of cysteine. Therefore, this study was designed to investigate the effect of germination and extrusion on the millet and cowpea protein and cysteine utilization using an *in vivo* study.

III MATERIALS AND METHODS

3.1 Materials

Chemicals and reagents – the following items were purchased from Sigma Chemical Co (St. Louis, MO): Tween 20 – P1379, bovine serum albumin – A4503, alkaline phosphatate conjugate monoclonal anti- rabbit IgG- A2556, and p-nitrophenyl phosphate- P7998; Vector Laboratories (Burlingame, CA): rabbit anti – Phytohemagglutinin IgG – AS 2300; Bio Rad Laboratories (Hercules, CA): Bio-Rad DC protein assay kit 500-0111; and Corning (New York, USA): 96 well EIA/RIA micro titer plate- 3369. All other chemicals and reagents were of analytical quality and were purchased from local vendors. California black eye cowpea and millet grains were purchased from local retailers and were stored in a cooler at 4°C.

3.2 Methods

3.2.1Germination

Germination of millet and cowpea followed the procedure described by Mubarak (2005) with slight modifications. Extraneous materials were removed from both millet and cowpea prior to soaking the grains in 95 % ethanol for 1 minute to provide surface infection. Both millet and cowpea were rinsed three times with distilled water and soaked in distilled water using the ratio of the grain to water of (1: 5 w/v) for 3 hours and 1 hour respectively. After soaking, both millet and cowpeas were germinated at room temperature in trays for 48 hours with 12 hrs of alternating durations of light and darkness. Water was sprayed on the trays at 12 hours intervals to wet the surface of the

grains. Millet had 93% germination and cowpea had 88%. Germinated millet and cowpeas were then extruded or cooked.

3.2.2 Non-germination

Non-germinated millet and cowpea were manually sorted to remove the extraneous material and soaked in ethanol for 1 minute. Both millet and cowpea grain were rinsed three times with distilled water and soaked in distilled water using the ratio of the grain to the water (1: 5 w/v) for 3 hours and 1 hour, respectively. After soaking, they were divided into two equal parts, one half to be extruded and the other half to be cooked in boiling water. The extruded portion was allowed to drain for 3 hours before extrusion, and the cooked half was cooked immediately.

3.2.3 Extrusion

Extrusion of germinated and non-germinated millet and cowpea was carried out using a JS30A twin-screw extruder (screw diameter 30 mm and length of 420mm manufactured by Quitong Chemical Industry Equipments Co, Ltd. Yantei, China). The barrel is divided into three zones: the feeding zone was unheated, the middle zone was heated to 115°C, and the exit end zone was heated to 130°C. The screw speed was 255 rpm and the die used during extrusion was 7mm in diameter. The extruded products were air dried prior to grinding.

3.2.4 Open Kettle Cooking and Drying

The germinated millet and cowpea were cooked separately in steam-jacketed kettles in distilled water. They were cooked for 45 minutes at 95°C. Cooked millet and cowpea were then dried at 50°C in a large forced air oven (Model K12395, Proctor & Schwartz Company, Philadelphia, PA, USA).

3.2.5 Grinding

After the drying, the cooked and extruded millet and cowpea were ground to pass through a 1.6 mm screen (Model D hammer mill manufactured by Fitzpatrick Company Chicago, IL, USA). These products were used to prepare diets that were subsequently used for the *in vivo* protein digestibility study.

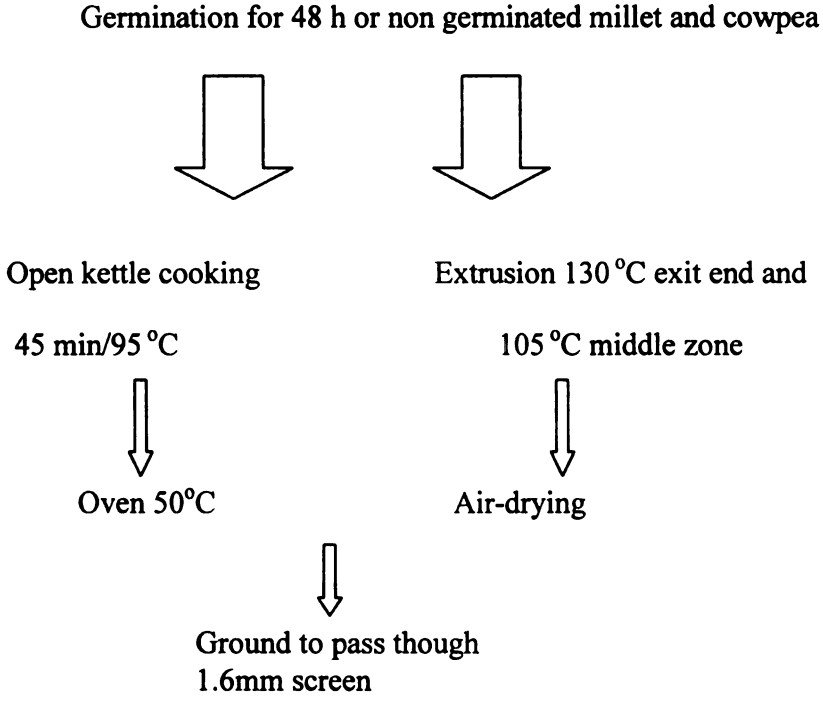


Figure 3.1: Flow chart diagram showing the processing of foods used in the *in vivo* study.

3.2.6 Protein Extraction

Proteins were extracted from the extruded and cooked samples using the extraction method described by Wong et al, (2004) with few modifications. The extracted proteins were separated into three fractions based on solubility in KCl and methanol. One g of fine flour was suspended in 4 mL of cold (4°C) KCl buffer (50mM Tris – HCl, 100mM KCl, 5 mM EDTA – pH 7.8). The samples were incubated on ice for 5 minutes with intermittent mixing (Vortex Genie 2, Scientific Industries, Inc, Bohemia, NY, USA) and centrifuged at 4 °C for 15 minutes at 14, 000 rpm (Sorvall RC-5B refrigerated super speed centrifuge, Sorvall Instrument, Newton, CT, USA). The supernatants were collected and the pellets were discarded. Five volumes of 0.1M-ammonium acetate in methanol was added to the supernatant and the mixture was incubated overnight at –20 °C. The samples were then centrifuged at 14, 000 rpm for 15 minutes at 4 °C to separate the methanol insoluble fraction (pellets) from the methanol soluble fraction (supernatant). The methanol insoluble fraction was resolubilized in the KCl buffer.

3.2.7 Protein Determination

The proteins in the methanol soluble and methanol insoluble fractions were determined by a modified Lowry method using reagents and instructions provided by Bio-Rad. The standard was prepared with bovine serum albumin. The modified Lowry method was used instead of the Kjeldahl method because of the ammonium that was used during the protein extraction. For total soluble protein, a modified Kjeldahl method was used (Yasuhara and Nokihara, 2001). One hundred μL of the total protein fraction described in section 3.2.6 was placed in the decomposing flask and 2 mL of concentrated sulfuric acid

(H_2SO_4) was added. The decomposing flask was placed on the heating unit (digesdahl digestion apparatus model 23130 – 20, by Hach Company , Loveland, CO, USA) that was preheated to 440°C. After the H_2SO_4 and protein extract boiled for 4 minutes, 4 mL of 30% Hydrogen Peroxide (H_2O_2) was added. This mixture was boiled for an additional 10 minutes to boil off excess hydrogen peroxide. The flask was removed from the heating unit and cooled to room temperature before the digest was diluted with 10 ml of polished water. Polished water is ultra pure water that has been passed through a C18 column- (#6544 500mg – Alltech Associate Inc, Dearfield, IL, USA). The digest was neutralized with approximately 15 mL of 2 M Sodium Carbonate (Na_2Co_3) and brought to 100mL with polished water. The digested samples were further diluted 2, 25, and 50 times with polished water for the colorimetric assay. One mL of n- hexane was pipetted into a test tube. Two mL of sample and 1ml of reagent 1 (Phenol – 1 g and Sodium Pentacyanonitrosyl Ferrate dihydrate – 5 mg, dissolved 100mL of polished water) was added. The test tube was shaken for a few seconds, and 1mL of reagent 2 (1mL of sodium hypo – chloride and 1.5g sodium hydroxide diluted in water to 100 mL) was added. The tube was gently mixed and heated at 50°C for 40 minutes. Absorbance was measured at 640 nm. A 2mM ammonium chloride (NH_4Cl_2) solution was used as a stock standard. The stock standard was diluted to 1:20 and the following amounts of ammonium chloride were concentrations were placed in series of test tubes: 0.04, 0.08, 0.12, 0.16 and 0.20μmoles.

3.2.8 Diet Formulation and Preparation

A 2X2X3 factorial study was designed to determine the effects of germination, extrusion and protein source on true protein digestibility and rat growth. The statistical design is shown in Table 3.1.

Table 3.1 Experimental design – a 2X2X3 factorial

DIET	EXTRUDED		COOKED	
	Germinated	Non-germinated	Germinated	Non-germinated
Millet	MGE	MNGE	MGC	MNGC
Cowpea	CGE	CNGE	CGC	CNGC
Millet + Cowpea	MCGE	MCNGE	MCGC	MCNGC

Positive control – (Soy concentrate as a source of 10% protein)

Metabolic protein – (Lactalbumin that provided 2.5% protein)

Raw millet – (millet as a source of 10 % protein)

Twelve diets were formulated according to the AIN 93G guidelines (Reeves, 1993) – Tables 3.2 – 3.6. The dietary products were as follows: Millet–non-germinated and cooked (MNGC), cowpea–non-germinated and cooked (CNGC), millet + cowpea– non germinated and cooked (MCNGC), millet–non-germinated and extruded (MNGE), cowpea–non-germinated and extruded (CNGE), millet + cowpea–non-germinated and extruded (MCNGE), millet–germinated and cooked (MGC), cowpea–germinated and cooked (CGC), millet + cowpea–germinated and extruded (MCGE), millet–germinated

and extruded (MGE), cowpea–germinated and extruded (CGE), millet + cowpea–germinated and extruded (MCGE).

The diets were formulated and processed in the laboratories of the Department of Food Science and Human Nutrition. The millet plus cowpea diets were blended to provide the maximum amino acid score for weanling rats. Three additional diets were included in the study: a positive control (Table 3.2) that used soy concentrate as a source of protein, a low protein diet used to estimate metabolic protein excretion, and a raw millet diet. All diets (except the metabolic protein diet) were formulated to contain 10 % protein. All other nutrients were provided at recommended levels and meet or exceeded known requirements for rat growth (Reeves et al, 1993). All diets were stored at refrigerated temperatures throughout the research period.

3.2.9 Protein Digestibility

Twenty one day old Male Sprague – Dawley rats were purchased from Harlan Sprague-Dawley, (Indianapolis, IN) and were housed individually in suspended stainless steel cages with wire bottoms. The temperature of the animal room was 22 ± 2°C, the relative humidity was 35 – 60% with alternating 12-hour periods of light and darkness. The rats were fed the AIN- 93 G diet (Reeves et al, 1993) for an acclimatization period of 4 days. The rats were weighed and those rats at the extreme end of the distribution curve were excluded from the study. The remaining animals were distributed into 15 groups of nine animals each. The differences in mean weight between any two groups did not exceed 0.6 g. Twelve groups were assigned to millet and cowpea experimental diets as shown in

table 3.1. The 13th group was a control group fed a modified AIN- 93 diet (Table 3.2, Reeves et al, 1993). The 14th group was fed a diet containing 2.5 % lactalbumin to estimate metabolic protein. The 15th group was fed raw millet. Food and water were provided *ad libitum.* The data collection period started after the fourth day of acclimatization period, and lasted for 21 days. Animal weights were recorded every third day during the 21 days period. Feces were collected for each rat on days 14 -17 and food intake was measured during the same time period. The following protein quality indices (apparent protein digestibility and true protein digestibility) were calculated from the data collected from the *in vivo* study:

$$\text{Apparent (N) digestibility (\%)} = \frac{\text{total N consumed- total fecal N}}{\text{Total N consumed}} * 100. \quad \text{(Eq. 1)}$$

$$\text{True (N) digestibility (\%)} = \frac{\text{total N consumed} - (\text{total fecal N- metabolic N})}{\text{Total N consumed}} * 100 \quad \text{(Eq. 2)}$$

$$\text{Amino acid score} = \frac{\text{amino acid content of feed type (test protein)}}{\text{Amino acid content in the reference protein}} \quad \text{(Eq. 3)}$$

The amino acid score of the feed type corresponds to the lowest ratio of essential amino acids.

Protein digestibility corrected amino acid score (PDCAAS) = amino acid score * true protein digestibility. (Eq. 4)

Table 3.2: Composition of positive control, metabolic protein and millet diets (g) used in the rat feeding study.

INGREDIENTS	POSITIVE CONTROL	METABOLIC PROTEIN	MILLET			
			NG-C[1]	NG- E[2]	G-C[3]	G-E[4]
Millet	0.0	0.0	2760.0	2760.0	2760.0	2760.0
Cowpea	0.0	0.0	0.0	0.0	0.0	0.0
Cornstarch	2803.9	2330.4	0.0	0.0	0.0	0.0
Lactalbumin	0.0	75.0	0.0	0.0	0.0	0.0
Soy concentrate	504.0	0.0	0.0	0.0	0.0	0.0
Soy bean oil	280.0	210.0	90.0	90.0	90.0	90.0
Fiber	212.0	231.6	0.0	0.0	0.0	0.0
Basal (table 3.5)	200.1	150.0	150.0	150.0	150.0	150.0
Totals	4000.0	3000.0	3000.0	3000.0	3000.0	3000.0

[1] NG-C, non germinated and cooked

[2] NG-E, non germinated and extruded

[3] G-C, germinated and cooked

[4] G-E, germinated and extruded

Table 3.3: Composition of cowpea diets (g).

INGREDIENTS	COWPEA			
	NG-C[1]	NG- E[2]	G-C[3]	G-E[4]
Cowpea	1160.4	1160.4	1160.4	1160.4
Corn starch	1661.3	1661.3	1661.3	1661.3
Lactalbumin	0.0	0.0	0.0	0.0
Soy concentrate	0.0	0.0	0.0	0.0
Soy bean oil	19.2	19.2	19.2	19.2
Fiber	9.0	9.0	9.0	9.0
Basal (see table 3.5)	150.0	150.0	150.0	150.0
Totals	3000.0	3000.0	3000.0	3000.0

[1] NG-C, non germinated and cooked

[2] NG-E, non germinated and extruded

[3] G-C, germinated and cooked

[4] G-E, germinated and extruded

Table 3.4: Composition of millet plus cowpea diets (g).

INGREDIENTS	MILLET + COWPEA			
	NG-C[1]	NG- E[2]	G-C[3]	G-E[4]
Millet	2167.6	2167.6	2167.6	2167.6
Cowpea	635.6	635.6	635.6	635.6
Cornstarch	976.5	976.5	976.5	976.5
Lactalbumin	0.0	0.0	0.0	0.0
Soy Concentrate	0.0	0.0	0.0	0.0
Soy bean oil	17.0	17.0	17.0	17.0
Fiber	3.2	3.2	3.2	3.2
Basal (table 3.5)	200.1	200.1	200.1	200.1
Totals	4000.0	4000.0	4000.0	4000.0

[1] NG-C, non germinated and cooked

[2] NG-E, non germinated and extruded

[3] G-C, germinated and cooked

[4] G-E, germinated and extruded

Table 3.5: Composition of basal mix used in all diets.

BASAL MIX	AMOUNT IN GRAMS
Mineral mix - minus calcium (AIN 93G)	1645.0
Vitamin mix (AIN 93G)	470.0
Calcium Carbonate ($CaCo_3$)	117.5
Choline Bitartrate	117.5
Butylated Hydroxy – Toluene (BHT)	0.7

3.2.10 Chemical Assays

Feces were dried for 3 days at room temperature, separated from the spilled food, and dried to a constant weight in an oven at 100°C. The feces were finely ground using a Science Ware-Micro Mill (Bel-Art products, Pequannock, NJ, USA). Total nitrogen in the diets and the feces was determined by Dumas method using a F P 528 Nitrogen Analyzer (LECO Corporation, St Joseph, MI, USA) and the crude protein content was calculated using the factor N (6.25).

3.2.11 Phytohemagglutinins Assay

Active phytohemagglutinins in the processed food samples were determined by a sandwiched enzyme - linked immuno sorbent assay (ELISA) as described by Boniglia et al 2003. One gram (1g) of dried and finely ground food sample was extracted in 20 mL of phosphate buffer solution (10mM phosphate buffer containing 150mM NaCl, pH7.2) by stirring overnight at room temperature. A 96 well easy wash micro- titer plate, no lid high binding certified, polystyrene plate was coated with 0.1 mL of porcine thyroglobulin (8ng/μL in 50 mM carbonate-bicarbonate buffer, pH 9.8) and incubated at 4^0C overnight. The treated microtiter wells were washed twice with PBT (phosphate buffer 10 mM, pH 7.2 containing 0.05% of Tween 20) and once with PBS. The coated micro titer wells were treated with 0.1 mL of PBS containing 0.05 % bovine serum albumin and incubated at 37 ^{0}C for 60 minutes. After incubation the plate was washed as previously described. The sample extracts (0.1mL) were loaded into the wells and incubated at 37^0C for 60 minutes and washed as described above. The micro titer plate was subsequently incubated with 0.1 mL of the following: 1) rabbit anti – Phytohemagglutinin IgG (diluted 1: 5000 in PBS containing 0.25% BSA); 2) alkaline phosphatate conjugate monoclonal anti- rabbit IgG (diluted 1: 10,000 in PBS containing 0.25%) and 3) p-nitrophenyl phosphate color development solution. Between treatments 1 and 2, the micro titer plate was incubated for 60 min at 37°C, rinsed twice with PBT and once with PBS. After the third incubation, 50 μL of 3M NaOH was added to each well to stop the reaction. Absorbance of the final solution was determined at 405 nm using a Microplate reader (Bio Rad, model 550, Hercules, CA, USA). Samples of millet and cowpea flour that had not received any

thermal treatments were also analyzed and were considered to contain 100% of active lectins for their respective grain.

3.2.12 Statistical analysis

Results are presented as mean ± standard deviation or standard error of the mean (SEM) as indicated. Data were subjected to a three-way analysis of variance (3 way - ANOVA) using the Prostat software, version 3.01 (Poly Software International Inc, Pearl River, NY, USA). Treatment differences were considered significant at $p \leq 0.05$. *Post hoc* analysis was done by the Fisher's LSD test.

IV RESULTS AND DISCUSSION

4.1 Total Soluble Proteins

Germination increased protein solubility for both millet and cowpea as expected. Wong et al, (2004) suggested that germination activates disulfide oxidoreductase to convert disulfide bonds to the sulfhydryl form, which in turn alters certain proteins so that they became more soluble in aqueous solutions. Germination increased the amount of soluble protein by 30 % and 22 % for millet and cowpea, respectively. Germination was expected to inactivate protease inhibitors by reducing disulfide bonds and in turn increase the availability of the sulfur containing amino acid cysteine.

4.2 Hemagglutinin Activities

Intake of active phytohemagglutinins interfere with the intestinal mucosal membrane brush border function by causing atrophy of the microvilli, and decreasing the absorption of most of the nutrients (Owen, 1996). Moreover, consumption of active phytohemagglutinin (for example insufficiently cooked beans) can be sufficiently serious to require hospitalization. Since hemagglutinin activity is so potentially toxic, it is important to determine hemagglutinin activity in any new process. Hemagglutinin activity was reduced to negligible levels by both open kettle cooking and extrusion (Table 4.1). There was no significant difference ($p \geq 0.05$) between extruded and cooked samples, suggesting that extruded legumes are as safe to eat as the cooked or boiled ones. Since the residual phytohemagglutinin activities were very low, it suggests that extrusion cooks the food sufficiently to render the food safe for human consumption.

Table 4.1:Cowpea feed preparation and hemagglutinin % reduction.

FEED TYPE	% REDUCTION IN HEMAGGLUTININ ACTIVITY[1]
Cowpea non- germinated and extruded	99 ± 2
Cowpea non-germinated and cooked	100 ± 0
Cowpea germinated and extruded	96 ± 7
Cowpeas germinated and cooked	98 ± 3.4
For millet and millet + cowpea diet, the hemagglutinin activities were reduced to undetectable levels	

[1] Mean ± Standard deviation, % reduction is based on raw cowpea, and raw millet for cowpea and millet diets respectively.

4.3 Amino Acid Score

Proteins that are deficient in one or more essential amino acid are considered as poor quality and this deficient is often reflected in the amino acid scores. Lysine is limiting in millet as in other cereal, and methionine is limiting in cowpea and other legumes. The Amino Acid Score (AAS) compares the amino acid profile of the protein in a food to the amino acid pattern required by a particular species at a particular age. For humans, the amino acid pattern required by 2 – 5 year olds (FAO, 1991) is used because it surpasses the amino acid requirement patterns of older children and adults. The AAS is calculated as a percentage or alternatively the AAS can be expressed in decimal 0 – 1.0 format. Any score above 100% is rounded to 100%. There is no benefit in consuming an amino acid above the amount required, because the body can only utilize up to 100%. Foods that have a score of less than 100% are considered to be limiting in one or more essential amino acids and may not satisfy the amino acids need for normal growth of 2 – 5 years old. The amino acid score for cowpeas alone (Table 4.2) was higher (0.51) than the amino acid score for millet (0.33) when using amino acid pattern for the laboratory rat as a standard (Table 4.2). Amino Acids listed in Tables 4.2 are from the USDA's National Nutrient Database for Standard Reference (www.nal.usda.gov/fnic/foodcomp - millet NDB No: 20031 and black eye pea NDB No: 16062). The most limiting amino acid in millet is lysine as with many cereals. Cowpea is limiting in sulfur containing amino acids as presented in table 4.2 and 4.3 for the laboratory rat requirement.

Table 4.2: Amino acid score for millet and cowpea in reference to the laboratory rat amino acid requirement pattern

	Amino Acid					
Amino Acid	Pattern for growing rats, mg/aa/g/protein	Pattern for millet, mg/aa/g/protein	Pattern for cowpea, mg/aa/g/protein	Ratio for millet	Ratio for cowpea	Ratio for millet plus cowpea[1]
Isoleucine	42	42.2	40.64	1.00	0.97	0.990
Leucine	62	127.8	76.6	2.05	1.24	1.724
Lysine	58	19.2	67.64	0.33	1.17	0.665
Sulphur aa *	50	39.3	25.29	0.79	0.51	0.674
Phenylalanine/ tyrosine**	66	83.5	90.68	1.26	1.37	1.308
Threonine	42	32	38.05	0.76	0.91	0.820
Tryptophan	12.5	10.7	12.32	0.86	0.99	0.908
Valine	50	52.5	47.66	1.05	0.95	1.011
Histidine	25	21.4	31.037	0.86	1.24	1.011

* Methionine and cysteine, less than 50 % can come from cysteine and ** less than 50% can come from tyrosine

[1] Millet 60 % and cowpea 40 %.

4.3: Amino acid score and limiting amino acids for millet and cowpea in reference to the laboratory rat amino acid pattern requirements.

Amino Acid	MILLET	COWPEA	MILLET + COWPEA[1]
Score	0.33	0.51	0.67
Rank [2]	Limiting Amino Acid		
First	Lysine	Methionine + Cysteine	Lysine
Second	Threonine	Threonine	Methionine + Cysteine
Third	Methionine + Cysteine	Valine	Threonine
Fourth	Valine + Histidine	Isoleucine	Tyrosine

[1] Sixty percent of the protein was derived from millet and 40% from cowpea.

[2] The amino acid listed in each row is the amino acid that is most deficient, second most deficient etc., for each column. The amino acid score is calculated using equation 3.

4.4 Protein Digestibility

Variable results have been reported for utilization of cereal and legume protein following cooking and extrusion. For example germination of legumes and cereals was reported to either have no effect, or to slightly increase protein utilization (Donangelo et al, 1995; Mbithi-Mwikya et al, 2002; Trugo et al, 2002 and Urbano et al, 2005). However it should be noted that published data on utilization of millet and cowpea protein are primarily limited to in vitro protein digestibility, and may not accurately reflect protein digestion by mammals.

In this study there was a small overall difference in protein digestion between germinated and non-germinated grains (Table 4.4), but the difference was not statistically significant ($p>0.05$). The lack of significant difference in overall protein digestibility between germinated and non-germinated grains is in part due to a significant interaction between open kettle cooking vs extrusion and germination vs non-germination. When the grains were cooked, germination improved protein digestion. However, when the grains were extruded, germination lowered protein digestibility (Table 4.5).

Table 4.4: Overall true digestibility (%) of protein as affected by germination, processing method and grain type.

TREATMENT
Germinated (75.4[a]) vs. non-germinated (73.9[a])
Cooked (65.8[a]) vs. extruded (83.6[b])
Millet (70.9[a]) vs. cowpea (80.9[b]) vs. millet + cowpea (72.2[a])

[1] Values are means. Values in the same row not sharing the same superscripts are statistically different ($p < 0.05$). The true digestibility (%) for the positive control was 90.2 $(0.5)^{h}$ and 87.1 $(1.0)^{gh}$ for raw millet protein. The complete results for the 3-way factorial ANOVA are shown in Appendix III.

Table 4.5: True digestibility (%) for protein in the various diets[1].

FEED TYPE	COOKED		EXTRUDED	
	Non-germinated	Germinated	Non-germinated	Germinated
Millet	52.6 $(1.5)^{a}$	57.3 $(2.4)^{b}$	89.9 $(0.9)^{h}$	83.6 $(0.9)^{efg}$
Cowpea	78.6 $(2.4)^{d}$	81.0$(1.3)^{def}$	79.7 $(1.7)^{de}$	84.4 $(2.1)^{fg}$
Millet + Cowpea	60.4 $(1.0)^{b}$	64.6$(1.1)^{c}$	82.3 $(0.7)^{def}$	81.6 $(1.1)^{def}$

[1] Values are means ± (SEM), N = 9, Values not sharing the same superscripts are statistically different ($p < 0.05$), The true digestibility (%) for the positive control was 90.2 $(0.5)^{h}$ and 87.1 $(1.0)^{gh}$ for raw millet protein. The true protein digestibility was calculated using equation 2.

There was an overall difference in protein digestibility depending on whether the grains were cooked vs extruded (Table 4.4). Moreover, there was a significant interaction between processing methods and grain type. It is evident from Table 4.5 that the most pronounced differences due to processing were found in the diet that contained millet, and that processing method had little effect on the protein digestibility of cowpea protein. Digestion of raw millet protein was high (87.1%) and similar to soy concentrate protein (90.2%).

Digestion of millet protein following extrusion was not different from raw millet. However cooked millet had a low true protein digestibility (55%, Table 4.4) compared to the extruded millet (87.1%). These results corresponded to the 52.9% protein digestibility that Kurien et al (1961) reported for cooked pearl millet. Both processing methods denature protein, but extrusion has limited moisture and higher heat for a short time compared to boiling in an excess of water. It is not known why boiling reduces millet protein digestion, but Eggum et al (1983) observed a similar decrease in protein digestibility by cooking sorghum.

Comparing the cooked cowpea to cooked millet, cooked millet had a lower ($p<0.05$) digestibility than cowpea, whereas raw and extruded millet yielded higher protein digestibilities than cowpea (Table 4.5). The millet + cowpea diet had higher protein than cowpea if the millet and cowpea were extruded whereas protein digestion was lower if millet and cowpea were cooked. These differences were most likely due to the millet rather than the cowpea.

The proportions of millet and cowpea in the millet plus cowpea diets were based on the maximization of amino acid scores. This approach produced the amino acid scores listed in table 4.3 and clearly show that a "complementary effect" was produced by blending millet with cowpea. Since not all amino acids in a food are available (e.g. not all protein is digested), multiplying the amino acid score of a particular protein by its protein digestibility coefficient produces the protein digestibility corrected amino acid score (PDCAAS). The PDCAAS is the best predictor of protein quality available today and the PDCAAS for the diets used in this study are shown in Table 4.6.

The millet-based diets have low PDCAAS because of the very low amount of lysine. The difference between cooked and extruded millet diets is due to the poor protein digestibility when millet is cooked. Moreover, there is little differences between the PDCAAS for cowpea and the cooked millet plus cowpea due to the poor digestion of millet protein when millet is cooked. The safe levels of protein intake for 3-4 years old children is 1.09 g/kg/day and decreases as the human body grow, and 0.75 g/kg/day for young adult of 19 and older. According to FAO/WHO/UNU, the PDCAAS at 65% will only be able to maintain the body and no growth support; this is specialty true for growing children.

Table 4.6: The Protein Digestibility Corrected Amino Acid Score (PDCAAS) of diets[1]

DIETS	PDCAAS (%) in reference to:	
	Weanling rat	2-5 yrs old
Millet non- germinated and cooked	17.49	17.49
Millet non- germinated and extruded	29.70	29.70
Millet germinated and cooked	18.81	18.81
Millet germinated and extruded	27.72	27.72
Cowpea non- germinated and cooked	40.29	79.79
Cowpea non- germinated and extruded	40.80	80.8
Cowpea germinated and cooked	41.31	81.81
Cowpea germinated and extruded	42.84	84.84
Millet + Cowpea non- germinated and cooked	40.47	40.2
Millet + Cowpea non- germinated and extruded	55.14	54.94
Millet + Cowpea germinated and cooked	43.28	43.55
Millet + Cowpea germinated and extruded	54.67	54.94

[1]The PDCAAS was calculated using equation 4

4.5 Food Intake

Feed intake has a major impact on growth because it affects the amount of energy and protein available for growth. The rat ate slightly more of the diet containing cooked foods to the diets containing extruded foods, but this difference was not significant (Table 4.7). Germination resulted in a significant increase in food intake (37.9g vs. 35.2g Table 4.7). The rats ate about 60% more of the millet + cowpea diet than either the diets containing millet or cowpea alone (Table 4.7).

There was a significant interaction between grain source and the type of processing. For millet + cowpea, open kettle cooking or extruding had no effect on food intake, but for millet or cowpea alone, rats ate 9% more of the cooked food than the extruded food (Table 4.8).

Both sensory and metabolic effects are known to influence food intake. The sensory attributes include the taste, physical characteristics while metabolic factor includes the plasma amino acid homeostasis and satiety. There is no significant difference in food intake regarding millet and cowpea individually. However, there is a significant difference in food intake ($p < 0.05$) regarding the combination of millet and cowpea.

It is well known that the type and amount of protein influences the food intake, and foods containing low and/ or imbalanced protein suppress the food intake, as reported by Mosha T C E, (2004). Reversible, food containing too high protein suppress food intake.

Table 4.7: Overall treatment effect on food intake (weights are in g)[1]

TREATMENT
Germinated (37.9^{b}) vs. non-germinated (35.2^{a})
Cooked (37.4^{a}) vs. extruded (35.7^{a})
Millet (29.95^{a}) vs. cowpea (31.8^{a}) vs. millet + cowpea (47.8^{b})

[1] Values are means. Values in the same row not sharing the same superscripts are statistically different ($p < 0.05$). The complete results for the 3-way factorial ANOVA are shown in Appendix V.

Table 4.8: Food intake for 3-day period (weights are in g)[1]

FEED TYPES	COOKED		EXTRUDED	
	Non-germinated	Germinated	Non-germinated	Germinated
Millet	$30.3(1.8)^{abc}$	$36.4\ (2.0)^{de}$	$25.6(0.8)^{a}$	$27.5\ (1.3)^{ab}$
Cowpea	$29.7\ (2.1)^{ab}$	$32.4\ (1.6)^{bcde}$	$30.3\ (1.4)^{abc}$	$34.8\ (3.0)^{cde}$
Millet + Cowpea	$47.1\ (1.3)^{f}$	$48.2\ (2.4)^{f}$	$47.8\ (1.9)^{f}$	$48.1\ (2.0)^{f}$

[1] Values are means ± (SEM), N = 9, Values in the same row not sharing the same superscripts are statistically different ($p < 0.05$). The food intake was 38.0g $(1.1)^{e}$ for the positive control and 27.6g $(1.4)^{ab}$ for raw millet.

4.6 Growth

All factors had a significant impact on growth but there were no significant interactions between the main factors - grain type, processing, or germination. The most marked effect was due to grain type. Rats fed the millet plus cowpea diet gained 1.7 times more weight than rats fed the cowpea diets and 3.8 times more weight than rats fed millet diets (Table 4.9). Likewise, rats fed cowpea grew twice as much as rats fed millet. Overall, rats fed germinated millet and cowpea grew 19% more than rats fed the non-germinated grains and rats fed cooked grains gained 9% more weight than rats fed the extruded grains. It also noteworthy that rats fed the millet plus cowpea diets, regardless of processing method or germination, all gained more weight than rat fed the positive control (soy concentration protein, Table 4.10). Growths for individual diets are presented in Table 4.10.

Table 4.9: Overall effect of germination, extrusion, and grain type on weight gain (g)[1]

TREATMENT
Germinated (51.1^{b}) vs. non-germinated (43.1^{a})
Cooked (49.1^{b}) vs. extruded (45.2^{a})
Millet (20.3^{a}) vs. cowpea (43.5^{b}) vs. millet + cowpea (77.5^{c})

[1] Values are means. Values in the same row not sharing the same superscripts are statistically different ($p < 0.05$). The complete results for the 3-way factorial ANOVA are shown in Appendix VII.

Table 4.10: Average weight gain for rats fed varying sources of protein (g)[1]

FEED TYPE	COOKED		EXTRUDED	
	Non-germinated	Germinated	Non-germinated	Germinated
Millet	19.3 (1.2)^{a}	28.9(1.7)^{b}	12.7 (1.2)^{a}	20.3 (1.1^{a}
Cowpea	40.2 (3.6)^{c}	47.3 (2.9)^{c}	39.5 (2.3)^{c}	47.1 (3.1)^{c}
Millet + Cowpea	74.3(1.8)^{e}	84.4 (4.8)^{f}	72.7 (4.1)^{e}	78.8 (1.7^{ef}

[1] Values are means ± (SEM), N = 9. Values in the same row not sharing the same superscripts are statistically different ($p < 0.05$). Weight gain for the positive control was 57.5g (1.7)^{d} and 16.0g (1.1)^{a} for raw millet .

Growth is dependent upon consuming sufficient energy and all nutrients required for growth. This research was designed so that the only limiting nutrient was protein. Moreover, the sulfur amino acids were most limiting in the cowpea and the millet plus cowpea diets. Therefore any small increase or decrease in available sulfur containing amino acids was expected to cause detectable differences in growth.

A complicating factor in this type of research is food consumption. Ideally, food consumption would be equal for all groups so that differences in growth would reflect only differences in bioavailability of the sulfur containing amino acids. As the PDCAAS becomes low, food intake is markedly depressed. The only small discrepancy in the data to this generalization is that the rats ate more cooked millet than extruded millet even though the PDCAAS's for the extruded millet were greater than the cooked millet.

Multiple linear regression of growth as the dependent variable and PDCAAS and food intake as the independent variables showed that 95% of the variation in growth is due to these variables. Figure 4.1 illustrate this relationship. Rats that ate the germinated cowpea and millet plus cowpea diets gained 13.6% more weight while only eating 5.6% more than rats eating the corresponding cooked grains.

Alternatively, rats eating the germinated cowpea and millet plus cowpea diets require less food ($p<0.05$) to gain 1g of body weight than rats eating the cooked grains. Likewise, rats eating the cooked diets gained more weight ($p<0.05$) per gram of protein consumed than rats eating the cooked cowpea and the millet plus cowpea diets.

These measures of efficiency strongly suggest that germination increased the bioavailability of cysteine, which in turn enhanced growth. Rats would have to be pair-fed to conclusively demonstrate that germination enhanced utilization of cysteine.

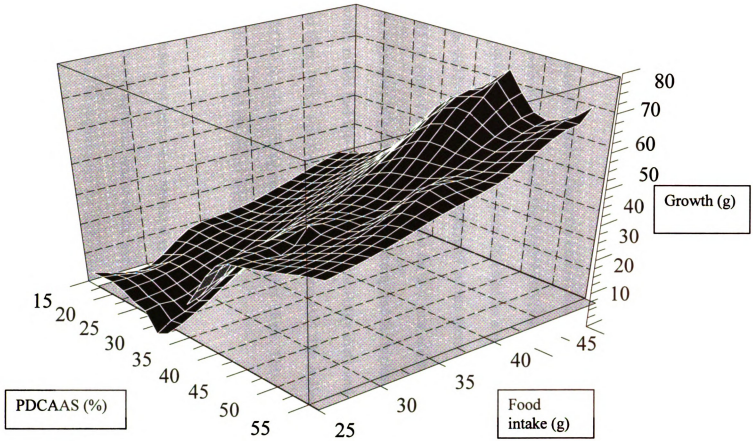


Figure 4.1 Growth (g) in weanling rats as affected by the protein digestibility corrected amino acid score %) of the diet consumed and food intake (g).

4.7 Applications to humans

Dietary protein is needed to maintain existing protein in the human body and to support normal growth. Adequacy of the protein component of the human diet depends upon the total amount of protein consumed, the pattern of essential amino acid and digestibility.

The PDCAAS (Table 4.6) index reflects the estimated ability of the test protein to meet the amino acid needs of an individual. The FAO/WHO/UNU recommended the use of the amino acid requirement pattern of 2-5 year old child as a reference for food meant for pre-school age children or older. The PDCAAS of the diets used in this study ranged from 17 to 85 % when the amino acid profile for 2-5 year old was used as a reference. The cowpea, regardless of germination or processing had the highest values ranging from 80-85%, while millet had the lowest scores. As for millet diets and the blend of millet plus cowpea diets, lysine was the most limiting amino acid. The millet and cowpea blend had high PDCAAS values (55%) compared to millet alone. This is due to the cowpea protein, which improved the amino acid profile.

When the laboratory rat amino acid profile was used as reference pattern, all products had serious deficiencies in essential amino acids, and the PDCAAS values ranged from 17 to 55 % for all foods. The requirement for sulfur containing amino acid requirement for rat are greater than for pre-school children, and growth of weanling rats is much proportionally faster than the growth of pre-school children. This difference explains the low PDCAAS values for foods when the rat's amino acid score was used. Since the requirements for methionine + cysteine and lysine for growing rats are much higher than

the requirement for preschool age children, the growth observed for the growing rats in this study are lower than what would have been if the diets has met all of the amino acids requirements for rats. This suggests that a combination of millet plus cowpea would not produce the complete amino acid requirement for growing rats.

A product that derived 66% of the protein from cowpea and 34% of the protein from millet would be a complete protein with 100% PDCAAS and would meet the amino acid needs for all people one year and older. This same product would also so be important in rehabilitating malnourished children. Addition of minerals, vitamins and fat source would make food nutritionally complete.

V CONCLUSIONS

This study showed that both the extruded and cooked blend of germinated millet and cowpea diets were of high quality and displayed great potential to support growth. The true protein digestibility values and growth response found in this study can be directly translated to humans. Germination had no apparent effect on protein digestion, however rats eating germinated grains ate more food ($p<0.05$) than they did if the grains were not germinated. Overall germinated grains produced significantly higher growth (51.1g) than non-germinated grains (43.1g). Extrusion processing yielded higher protein digestibility (83.6%) than if the grains were not germinated (75.4%). This indicates that the type of thermal processing is more important than germination when it comes to protein digestibility. Activation of oxidoreductases in millet and cowpea grains improves protein utilization for growth probably by increasing the bioavailability of the cysteine. Appropriate combination of 52% millet and 48% cowpea offers great potential for growth, because a high quality protein, especially for children and those who are HIV+, is needed. On the other hand extrusion processing can be used to produce more versatile and more digestible (83.6%) foods than open kettle cooking method (65.9%). Extrusion cooking has the potential to replace the traditional labor intensive, energy and time consuming cooking methods used in the production of many traditional foods. Extrusion presents a valuable opportunity for overcoming food insecurity in developing world, because extruded foods maintain extended shelf life and are ready-to-eat foods or they can be reconstituted in hot water. Use of this extrusion technology will save time and energy, which is a major constrain in traditional method of cooking food in boiling water.

VI SUGGESTIONS FOR FURTHER RESEARCH

In this study, bioavailability of cysteine was measured indirectly. The data strongly suggested that germination improved cysteine bioavailability, but it cannot be concluded with 100% confidence that germination was the only factor increasing growth, since small differences in food intake may be the most important factor in improved growth. Therefore a separate study is needed where food intake is closely controlled to evaluate the difference in the protein (cysteine) bioavailability in germinated and non-germinated millet plus cowpea. Further protein in germinated food could be hydrolyzed with immobilized proteases to determine if more of the free amino acid cysteine is produced. Further, a separate study is needed to investigate why conventional open kettle cooking significantly reduces protein digestibility of millet, but not cowpea.

APPENDICES

VII APPENDICES

Appendix I: The hemagglutinin levels of different diets as read at absorbance 740nm.

DIET	ABSORBANCE[1]
Millet non- germinated and cooked	0.066 ± 0.066
Millet non- germinated and extruded	0.078 ± 0.066
Millet germinated and cooked	0.074 ± 0.026
Millet germinated and extruded	0.083 ± 0.004
Cowpea non-germinated and cooked	0.081 ± 0.004
Cowpea non-germinated and extruded	0.090 ± 0.002
Cowpea germinated and cooked	0.097 ± 0.013
Cowpea germinated and extruded	0.093 ± 0.051
Raw Millet	0.142 ± 0.004
Raw Cowpea	0.234 ± 0.031

[1] Means ± Standard deviation

MNGC- millet non germinated and cooked, CNGC- cowpeas non germinated and cooked, MCNGC- millet + cowpeas non germinated and cooked, MNGE- millet non germinated and extruded, CNGE- cowpea non germinated and extruded, MCNGE- millet + cowpeas non germinated and extruded, MGC- millet germinated and cooked, CGC- cowpeas germinated and cooked, MCGC – millet + cowpeas germinated and cooked, MGE- millet germinated and extruded, CGE – cowpeas germinated and extruded, MCGE –millet + cowpeas germinated and extruded, RM- raw millet.

Appendix II: Protein Digestibility (%) for rats fed millet, cowpea and millet + cowpea diets

PROTEIN DIGESTIBILITY (%) OF DIETS												
Rats	MNGC	CNGC	MCNGC	MNGE	CNGE	MCNGE	MGC	CGC	MCGC	MGE	CGE	MCGE
1	54.06	79.02	64.54	84.47	79.01	79.07	56.90	87.44	63.44	80.91	83.97	86.18
2	50.87	74.21	62.01	89.81	83.65	80.09	58.04	81.08	59.54	84.20	90.37	79.23
3	54.42	83.06	58.44	88.10	73.01	82.93	53.60	79.04	66.47	82.96	86.31	75.91
4	47.61	82.06	57.23	92.31	83.76	83.63	60.49	84.34	64.71	81.35	79.35	78.40
5	56.40	81.16	63.29	90.07	79.72	84.00	47.66	78.22	63.67	84.63	85.50	83.05
6	46.31	79.54	58.73	91.69	71.86	82.46	55.18	75.20	68.11	82.91	88.51	81.70
7	50.24	85.77	62.71	91.86	85.99	84.59	53.32	80.77	70.62	80.61	69.96	82.57
8	60.08	61.25	60.74	91.29	84.14	79.86	59.59	84.74	61.38	89.83	84.76	84.95
9	56.58	80.98	56.05	89.25	75.94	83.91	71.37	78.52	63.82	84.70	90.51	83.79

Appendix III: Three way factorial ANOVA on protein digestibility

Factorial ANOVA Statistics Report on protein digestibility

Factorial ANOVA

Source	DF	SumOfSq	MeanOfSq	F-Value	P-Value
C1	1	724.889	724.889	6.703	0.011
C2	1	1742.430	1742.430	16.113	0.000
C3	2	57909.337	28954.669	267.749	0.000
Main Eff.	4	60376.656	15094.164	139.578	0.000
C1*C2	1	19.423	19.423	0.180	0.673
C1*C3	2	128.965	64.482	0.596	0.553
C2*C3	2	96.815	48.408	0.448	0.640
C1*C2*C3	2	12.040	6.020	0.056	0.946
Error	96	10381.558	108.141		
Total	107	71015.457	663.696		

Appendix IV: Food Intake for rats fed varying millet, cowpea and millet + cowpea diets

RAT #	FOOD INTAKE (g)											
	MNGC	CNGC	MCNGC	MNGE	CNGE	MCNGE	MGC	CGC	MCGC	MGE	CGE	MCGE
1	32.651	35.21	41.72	30.7	30.83	52.55	38.66	28.91	40.71	32.55	30	48.7
2	33.01	22.6	49.13	23.67	25.14	50.11	36.95	26.9	51.49	26.02	35.21	46.7
3	33.64	24.92	47.76	26.08	26.48	44.35	35.52	36.86	38.4	29.87	26.26	48.56
4	24.89	23.29	52.65	26.25	30.52	35.36	35.53	30.58	53.35	20.02	30.9	44.04
5	30.56	28.51	41.61	24.1	29.15	43.22	50.36	27.95	53.72	25.83	26.03	49.26
6	21.42	36.62	48.36	27.7	26.43	49.9	28.53	38.29	55.87	27.1	39	35.16
7	37.47	23.8	50.92	27.92	31.53	49.79	34.85	36.8	44.09	28.88	55.38	55.85
8	24.76	35.42	46.48	28	34.51	51.64	35.3	37.34	56.15	25.25	38.43	52.22
9	34.59	36.77	45.56	24.95	38.34	53.47	31.6	27.8	40.19	31.87	31.79	52.21

Appendix V: Three way factorial ANOVA on food intake

Factorial ANOVA Statistics Report for food intake

Source	DF	SumOfSq	MeanOfSq	F-Value	P-Value
C1	1	61.111	61.111	1.917	0.169
C2	1	178.332	178.332	5.595	0.020
C3	2	6834.729	3417.364	107.213	0.000
Main Eff.	4	7074.172	1768.543	55.485	0.000
C1*C2	1	13.356	13.356	0.419	0.519
C1*C3	2	318.388	59.194	4.994	0.009
C2*C3	2	48.707	24.353	0.764	0.469
C1*C2*C3	2	54.632	27.316	0.857	0.428
Error	96	3059.942	31.874		
Total	107	10569.196	98.778		

Appendix VI: Growth for rats fed varying millet, cowpea and millet + cowpea diets

RAT	GROWTH (g)											
#	MNGC	CNGC	MCNGC	MNGE	CNGE	MCNGE	MGC	CGC	MCGC	MGE	CGE	MCGE
1	22.1	39.3	71.3	13.7	39.1	86.8	33.4	40.3	74.3	18.9	44.9	81.7
2	22.2	33.7	86.4	11.5	37.1	77.1	29.4	38.4	84.1	20.5	55.6	78
3	22.4	42.8	-	8.6	32.7	64.1	32.8	55.7	78.2	24.9	31.2	90.6
4	18.3	28.7	81.9	16.2	38.3	52	31.2	42	100.4	15.4	45.8	76.6
5	16.3	37.6	74.9	5.1	31.2	60.8	31.8	40.8	97.2	17.9	36.8	83.9
6	13.3	50	74	14.8	34	75.8	25.3	62.5	99.5	19.6	61.2	-
7	22.4	24	82.9	14.6	46.5	71.7	23.6	54.9	74.8	20.4	51.9	82
8	14.3	49.1	76.3	15.1	44.1	75.3	33.2	49.8	93.4	18.7	50.9	74.1
9	22	57	72.6	14.7	52.4	91	19.4	41.1	57.7	26	45.5	83.4

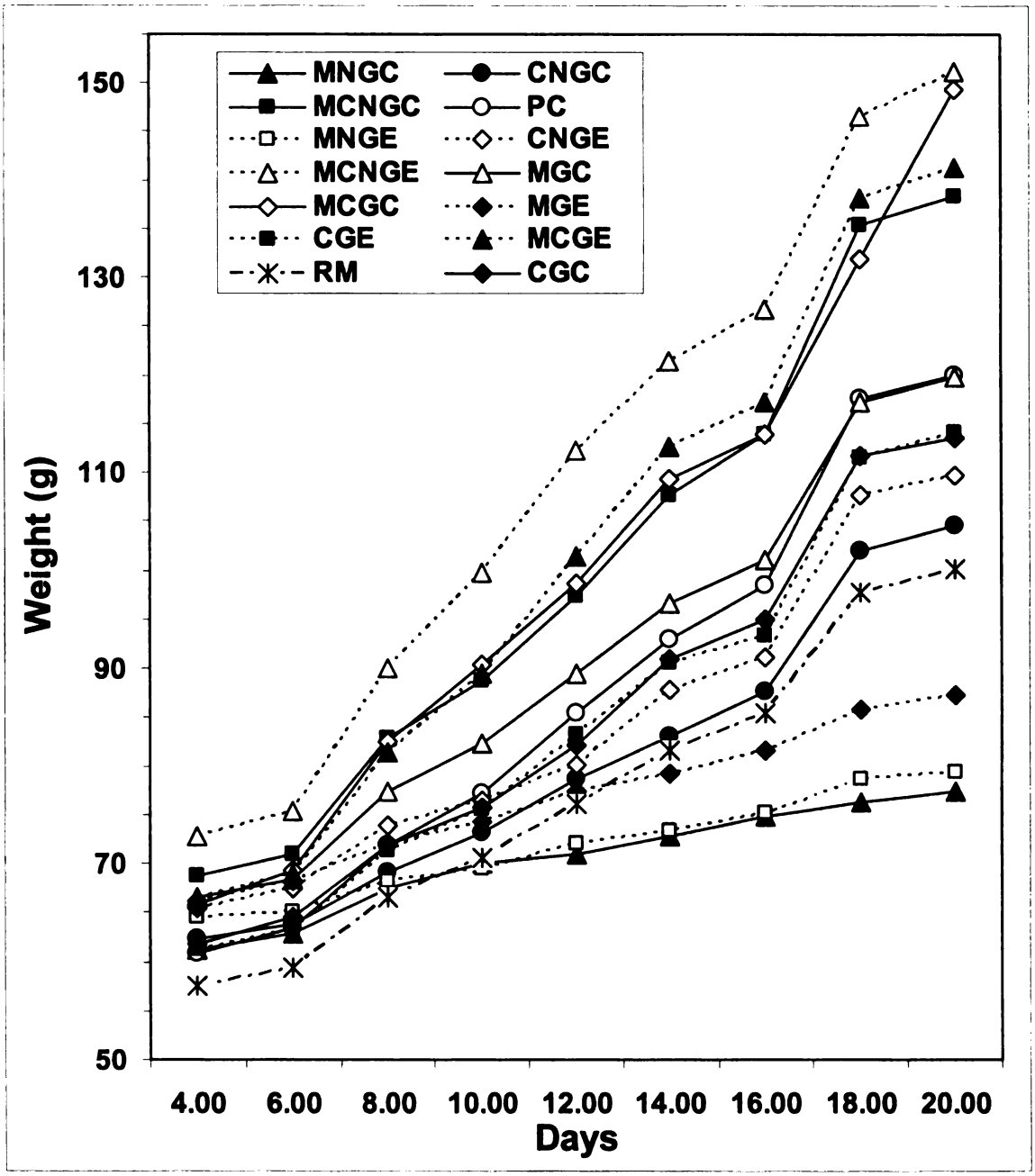
Appendix VII: Three way factorial ANOVA on growth

Factorial ANOVA Statistics Report on growth

Factorial ANOVA

Source	DF	SumOfSq	MeanOfSq	F-Value	P-Value
C1	1	724.889	724.889	6.703	0.011
C2	1	1742.430	1742.430	16.113	0.000
C3	2	57909.337	28954.669	67.749	0.000
Main Eff.	4	60376.656	15094.164	139.578	0.000
C1*C2	1	19.423	19.423	0.180	0.673
C1*C3	2	128.965	64.482	0.596	0.553
C2*C3	2	96.815	48.408	0.448	0.640
C1*C2*C3	2	12.040	6.020	0.056	0.946
Error	96	10381.558	108.141		
Total	107	71015.457	663.696		

Appendix VIII: Growth pattern for rats fed millet, cowpea and millet + cowpea diets



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