FORMULATION AND DEVELOPMENT OF PEANUT-BASED READY-TO-USE THERAPEUTIC FOODS FOR MALNOURISHED PREGNANT WOMEN IN MALI

by

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(Under the Direction of Jinru Chen)

ABSTRACT

Malnutrition is a condition affecting much of the developing world that results from diets low in nutrients and energy needed by the body. The treatment option used often in children involves the use of Ready-to-Use Therapeutic Foods (RUTFs), which are nutrient and energy dense foods that require no additional preparation. Most RUTFs currently used are similar and consist of ground peanuts, powdered milk, sugar, oil and fortified with a vitamin/mineral premix. Children are not the only group affected by malnutrition, however, with pregnant women being at a high risk of becoming malnourished, which affects both mother and infant. Producing the RUTF formulation locally requires powdered milk to be imported, increasing costs and making the RUTF less accessible for those in need.

The goal of this research was to use formulation software to develop plant-based RUTFs for pregnant women in Mali using largely local ingredients. Once formulated, six RUTF formulations containing peanuts, cowpeas, millet and rice or barley (as koji) were processed using simple technologies commonly available in Mali. The proximate nutrients for the RUTFs were analyzed and compared to the predicted values. Actual values were found to be similar to the software predicted values, with actual energy values having the greatest difference, ranging
from 11.90-19.70%. The RUTFs were processed using techniques including roasting, decorticating, boiling, milling, enzymatic hydrolysis and heat sterilization. Once processed, seven sensory characteristics of the RUTFs were determined using a descriptive panel.

As another option to help with malnutrition, probiotics have been shown to help improve physiologic properties that are impaired in the host intestinal tract. The survivability of *Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus acidophilus, Bifidobacterium lactis* and a five-strain mixture with rice or potato maltodextrin in capsules stored at 37, 25 and 4 °C was observed in the present work. Probiotics stored at 4 °C were found to survive at higher population levels compared to those stored at 25 or 37 °C. This project looks at malnutrition from a different perspective, focusing on using formulation software, local ingredients and simple processing technologies to produce RUTFs to treat malnutrition in pregnant women.

INDEX WORDS: Malnutrition, Ready-to-Use Therapeutic foods, Mali, Pregnant women, Probiotics, Descriptive sensory analysis
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This dissertation is dedicated to my parents, family, “adopted family” and friends who have supported me and encouraged me through this entire process.
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CHAPTER 1
INTRODUCTION

Malnutrition is a condition that results from an unbalanced diet that does not provide the energy and essential nutrients needed by the body. While the number of people suffering from malnutrition world-wide has decreased between the years of 1990 and 2012, the number of malnourished people in Sub-Saharan Africa has increased from 17% to 27% of the population during this same time period (FAOa 2012). All age groups are affected by malnutrition, but infants, young children and women, specifically pregnant and lactating women, are the most nutritionally vulnerable groups due to high physiological nutrient requirements (Lartey 2008).

The short and long term consequences of malnutrition can have a major impact on the population of a community and country. A person suffering from malnutrition often has a weakened immune system and a higher susceptibility to disease and infection, which can ultimately end in death without proper treatment (Müller and Krawinkel 2005; Walton and Allen 2011). Malnutrition also reduces a person’s quality of life as long term consequences from untreated malnutrition include mental developmental problems, educational underachievement and a reduced ability to work. Long-term outcomes affect not only the individual, but their family as well since a reduced ability to work creates a cycle of household hunger and poverty due to fewer people in the home being able to provide for the family (Onis and others 2000; Walton and Allen 2011; FAOb 2012).

In several countries in Sub-Saharan Africa, 10-19% of the female population has been noted as suffering from malnutrition (Black and others 2008; Lartey 2008). Mali, a West
African country in Sub-Saharan Africa, has a young population with a life expectancy at birth of 53 years that is devastated by climate and civil wars (CIA 2012). Current conflict in northern Mali has put an estimated 560,000 children less than five years old at risk of malnutrition. In August of 2012, 16,334 children and 2,329 pregnant/lactating women were enrolled in treatment programs for moderate acute malnutrition (OCHA 2012).

Malnutrition during pregnancy also has serious short and long term consequences since the effects of maternal malnutrition are intergenerational including maternal morbidity and mortality (Lartey 2008; Victora and others 2008). Pregnancy malnutrition has been linked to intrauterine growth restriction, which is a risk factor for neonatal conditions including low birth weight and deficits in lean body mass in fetal composition (Black and others 2008; Lartey 2008; Victora and others 2008).

Proper treatment of malnutrition is necessary to prevent the short and long term effects of malnutrition. The traditional treatment for malnutrition requires hospitalization and feeding with a liquid diet consisting of milk powder, water, vegetable oil and sugar fortified with vitamins and minerals (Ashworth and others 2003; Diop and others 2003; Ciliberto and others 2005; Manary and Sandige 2008; Walton and Allen 2011). This liquid diet is highly susceptible to microbial growth, making hospitalization necessary to ensure the liquid feeds are prepared under hygienic conditions with sterile water (Diop and others 2003).

This treatment plan is successful in helping patients recover from malnutrition, but is not feasible to set up and sustain outside of emergency situations. Limitations with the liquid feeds resulted in a new model of treatment for malnutrition which involved home treatment using ready-to-use therapeutic foods (RUTFs) (Briend and Collins 2010). RUTFs are energy dense foods that resist microbial growth and contamination that can be consumed directly without
further preparation or the addition of water. The RUTF commonly used is made from peanut butter, dried skim milk, oil, and sugar fortified with a vitamin/mineral supplement (Briend and others 1999). The main advantage of RUTFs is that microbes cannot grow and no further preparation is needed, which allows for home treatment. Historically, the RUTF was designed for children under the age of five. However, they are not the only group vulnerable to malnutrition and breaking the cycle of malnutrition requires focusing on different target groups, such as pregnant women.

One commercially available RUTF is PlumpyNut®, which is manufactured by the Nutriset Corporation (Nutriset 2013). This product must be imported which can be costly and not feasible in many developing countries. In order to reduce the cost of the RUTF, research has focused on producing RUTFs locally. Using the same ingredients as PlumpyNut®, Manary (2006) made a RUTF in Malawi using a bakery mixer and then filling the product into plastic containers. However, this locally made RUTF still contained powdered milk, which is a product that must be imported and adds to the total cost of the product (Dibari and others 2012). As a way to reduce costs, new products have utilized plant-based commodities that have been suggested to deliver the nutrition necessary. More recently, plant-based RUTFs for adults have been explored. Dibari and others (2012) have formulated a RUTF prototype for wasted children and adults in East Africa using soybeans, corn, sorghum, oil, and sugar that has a nutrient profile similar to PlumpyNut®.

One technique, known as linear programming has recently been applied to the development of RUTFs. This technology has been utilized in developing animal feed rations and has been applied to development of food for humans. Linear programming is used to determine the best solution to a problem in the form of a mathematical model based on a set list of
requirements (Al-Deseit 2009). The use of linear programming in formulating foods for human consumption has proved beneficial in formulating nutrient-dense foods using the ingredients available. In Sub-Saharan Africa, cereals are the main source of calories in the diet, but legumes are also available, and when the two are combined in a 50:50 ratio, a complete protein source can be created (Mensa-Wilmot and others 2003).

Along with using locally available ingredients, simple processing techniques also help reduce the total cost of a RUTF. Manary (2006) used simple processing techniques, mainly mixing of the ingredients (using pre-ground peanuts) hand filling to keep production costs low when producing a RUTF in Malawi. Identifying the processing technologies available in a given country would allow for the RUTFs to be produced in a country while minimizing costs. The processing methods often utilized in households in developing countries include boiling, dehulling, pounding, soaking, germination, enzymatic hydrolysis and fermentation (Nout and Ngoddy 1997; Hotz and Gibson 2007). These techniques, alone or combined, can be used to produce high quality, nutritionally dense, microbiologically safe RUTFs.

The effects of malnutrition are extensive within the body, including impairing nutrient absorption in the intestinal tract and impairing the immune system. In protein energy malnutrition, a majority of the host defense mechanisms are breached, depending on the severity of the protein deficiency in relation to energy, which allows microbes to invade the hosts intestinal tract producing clinical infection that is often more severe and prolonged (Cano and others 2002). Studies looking at the effects of probiotics on intestinal recovery from malnutrition suggest that there are positive benefits from probiotic ingestion, including higher counts of needed immune cells (Cano and others 2002; Galdeano and others 2011).
The objective of this work was to develop plant-based, ready-to-use therapeutic foods for malnourished pregnant women in Mali using computer software to create formulations that provide the nutrient density needed for treatment of malnutrition. Once formulated, a processing scheme was developed using simple processing methods and the products were characterized based on the nutrient, physical, and flavor profiles. The stability of potential probiotics and vitamins to enhance the treatment value of the RUTFs was also observed.

**Experimental Approach**

In order to overcome the limitations of the previously available RUTFs, an alternate strategy has been developed. This research features the use of indigenous commodities available in the selected target country to produce RUTFs catering to malnourished pregnant women in the population to prevent and reduce malnutrition.

The focus of this research is to develop semi-liquid RUTFs that use only local ingredients. Although a semi-liquid RUTF has obvious challenges related to shelf life and microbiological safety, it is easier to swallow and easier to digest compared to a thick paste. Using least-cost computer software, formulations meeting desired specifications were developed. Ingredient nutrient profiles and prices along with specific nutritional requirements of pregnant women were entered into least-cost computer formulation software and RUTF formulations based on cost and nutritional needs have been developed.

Simple processing techniques that can be easily adopted in the chosen target country were selected to produce the RUTFs. Indigenous commodities are generally comprised of cereals, legumes, roots and tubers, all of which contain significant amounts of starch which results in viscous pastes after necessary thermal processing (Nout and Ngoddy 1997).
Therefore, to produce semi-liquid RUTFs, enzymatic hydrolysis of starch can be used to decrease the viscosity of the product.

One possible source of starch hydrolyzing enzymes is fermented grains which contain enzymes such as α-amylases (BeMiller 2007). One fermented grain product that has been used as an enzyme source is koji. Koji is a fungal solid-state fermentation that has been used for centuries in Asian cultures to produce products including soy sauce, miso and sake (Watanabe and others 1998). Koji is made by steaming grains such as rice, barley, wheat or soybeans and then inoculation with the spores of Aspergillus oryzae. The inoculated grains are incubated under specific temperatures and humidities for a given amount of time, allowing the A. oryzae spores to grow and penetrate into the grains, producing multiple enzymes including α-amylases (Machida and others 2008). Following fermentation, the enzymes in koji can be used immediately to produce the desired final products. In the present research, rice and barley koji will be produced to utilize the enzymes in the koji to hydrolyze the starch and possibly the proteins in the product. Protein hydrolysis is desirable to produce a more easily digested RUTF. However, the formation of bitter peptides is also a possible challenge because it creates an unpleasant taste in the product. The koji produced for the present work provided an inadequate level of proteases, therefore a commercial protease, bromelain, found in pineapple, was utilized in the present work.

Producing nutritionally complete, microbiologically safe, shelf stable RUTFs is a crucial piece of this research. Processing techniques that are sufficient to destroy pathogenic bacteria while still being readily available in the target country, specifically thermal processing methods, were used. Packaging strategies to maintain microbiological safety were also investigated. One option is to retort the semi-liquid formulas in glass bottles or jars. Nutritional properties of the
processed RUTFs were characterized following processing to compare software predicted nutrient values to the actual values. The efficacy of the heat treatment was verified through microbial testing. The flavor and texture profiles of the RUTFs were determined through the use of descriptive sensory analysis.

Another portion of the overall strategy is to add the vitamin/mineral premix and probiotics to the RUTFs immediately after the RUTF is opened before it is consumed. The vitamin/mineral premix and the probiotics are susceptible to heat and the addition of these supplements before thermal processing would result in reduced quantities remaining in the RUTFs. As a way to overcome this problem, the vitamin/mineral premix and the probiotics were filled into separate hypromellose capsules, which were then be placed into polypropylene straws. The straws will be heat sealed once filled with the capsules, and attached to the container of RUTF, with the capsules being added just before consumption.

References


CHAPTER 2

LITERATURE REVIEW

Malnutrition

Malnutrition is a broad term referring to an unbalanced food intake in the diet that results in deficient (under nutrition) or excessive (over nutrition) intake of macro- and micronutrients (FAO Food Security 2012; FAO Hunger Portal 2012). In this present work, malnutrition will be used to describe the consequences of prolonged levels of low food intake and/or poor absorption of the food consumed, often resulting in protein and energy deficiency (FAO Hunger Portal 2012).

The number of people suffering from malnutrition, with a focus on developing countries, has decreased from 1,000 million (19% of the population) in 1990-1992 to 868 million people (12% of the population) in 2010-2012 (FAO Hunger Portal 2012). Of the total 868 million people affected, the two regions with the largest number of people suffering from malnutrition are Southern Asia, 304 million, and Sub-Saharan Africa, 234 million (FAO Hunger Portal 2012). In Sub-Saharan Africa malnutrition has shown the opposite trend of global malnutrition, with the number of malnourished people increasing from 170 million (17%) to 234 million (27%) of all malnourished people globally (FAOc 2012).

Malnutrition is a condition that can have short and long term effects that has a major impact on the population of a community and country. Ultimately, malnutrition can result in morbidity, as a person suffering from malnutrition has a weakened immune system and therefore a higher susceptibility to disease and infection (Müller and Krawinkel 2005; Walton and Allen...
One infectious disease that malnourished individuals are more susceptible to is diarrhea. Results from studies suggest that while diarrheal illnesses can increase a children’s susceptibility to malnutrition, it has also been suggested that malnourished children have an increased incidence of diarrhea, with the episodes, duration and total days with diarrhea being greater in malnourished children compared to healthy children (Guerrant and others 2008).

A person’s quality of life can also be substantially reduced, with developmental problems, educational underachievement, and a reduced ability to work being potential long-term outcomes of malnutrition. These long-term outcomes affect not only the individual, but their family as well since a reduced ability to work creates a cycle of household hunger and poverty when less people in the home can provide. The person’s community and country are also affected by a reduced work capacity as it impacts the economy and reduces national development (Onis and others 2000; Walton and Allen 2011; FAOb 2012).

**Categories of malnutrition**

The pattern of malnutrition in any community and region must be identified since the type and severity of malnutrition influence the preventative and treatment measures that are appropriate (Waterlow 1972). Malnutrition can be divided into two general types, macro-and micro-nutrient deficiencies (Müller and Krawinkel 2005). The severity of malnutrition is also split into two levels, acute and chronic malnutrition (Collins and others 2006).

**Macronutrient malnutrition**

Macronutrient malnutrition occurs when a diet is frequently deficient in protein, fat or carbohydrates, leading to protein-energy malnutrition (Müller and Krawinkel 2005). Protein-energy malnutrition is further divided into two categories, kwashiorkor and marasmus, with kwashiorkor associated with a higher mortality rate than marasmus (Waterlow 1972; Müller and
Krawinkel 2005; Manary and Sandige 2008; Prada and others 2011). Kwashiorkor is usually associated with a diet deficient in protein but provides a relatively adequate energy supply while marasmus is due to an overall deficiency in both energy and protein (Waterlow 1972). A person with kwashiorkor presents with nutritional oedema, which is pitting oedema of the feet that shows no other identifiable cause (Walton and Allen 2011). Classic physical symptoms of kwashiorkor also include growth retardation, psychic changes including anorexia and apathy, changes to hair and skin color, lethargy, dermatitis (skin lesions), anemia, hepatomegaly (enlarged liver) and early death (Waterlow and Scrimshaw 1957; Müller and Krawinkel 2005; Walton and Allen 2011). Marasmus, also referred to as severe wasting, is diagnosed by the loss of muscle and subcutaneous fat due to the endogenous mobilization of available energy and nutrients in the body (Müller and Krawinkel 2005; Walton and Allen 2011).

Physiologically, kwashiorkor and marasmus disrupt normal mechanisms in the body leading to ‘reductive adaptation’, which mobilizes energy and nutrient reserves, allowing the body to survive on minimal energy (Collins and others 2006; Walton and Allen 2011). The body’s adaptation to minimal energy impairs nutrient digestion and absorption through reduced production of enzymes and acid, atrophy of the villi in the intestines, decreased gut motility and limited ability to respond to stressors such as infections (Müller and Krawinkel 2005; Collins and others 2006; Walton and Allen 2011).

The severity of malnutrition, specifically in children less than five years of age, is determined using weight and height or mid upper arm circumference (MUAC) measurements (Collins and others 2006; Manary and Sandige 2008). Acute malnutrition, also referred to as wasting, is defined using weight-for-height measurements and is divided into two levels of severity, moderate acute and severe acute malnutrition (SAM) (Collins and others 2006).
Moderate acute malnutrition is the result of a weight-for-height z-score between two and three standard deviations below the mean value. SAM is defined as a weight-for-height z-score greater than three standard deviations below the mean or a MUAC <110 mm (Collins and others 2006; Collins 2007; Manary and Sandige 2008). Either form of acute malnutrition without nutritional oedema is often referred to as marasmus while SAM in the presence of oedema is called kwashiorkor (Manary and Sandige 2008). Globally, approximately 60 million children less than five years of age suffer from moderate acute malnutrition while 13 million suffer from SAM, with 9% of the children with SAM living in Sub-Saharan Africa (Collins and others 2006).

Chronic malnutrition, also termed stunting, is determined using height-for-age measurements. Height-for-age scores indicate chronic growth restrictions, often the result of long-term malnutrition (Waterlow 1972; Black and others 2008). In 2000, the prevalence of stunted children in West Africa was an estimated 34.9%, indicating long-term malnutrition (Onis and others 2000).

**Micronutrient Malnutrition**

Deficiencies from micronutrients are prevalent in developing countries where the diet has little variety, even if the food supply is adequate in meeting the needed energy requirements (Kennedy and others 2003). Micronutrient deficiencies, often referred to as “hidden hunger”, can go unnoticed in a community or region if a person does not exhibit the classic symptoms, even if growth, immune function and cognitive development are delayed (Bhaskaram 2002; Kennedy and others 2003). Worldwide, at least 2 billion people are affected by micronutrient deficiencies, with many suffering from multiple micronutrient deficiencies (Kennedy and others 2003; Müller and Krawinkel 2005; Ramakrishnan and Huffman 2008).
The three micronutrients causing the most widespread forms of micronutrient malnutrition with serious health consequences are vitamin A, iron and iodine (Kennedy and others 2003; Muller and Krawinkel 2005; Ramakrishnan and Huffman 2008). A deficiency in vitamin A can result in an impaired immune system and poor vision, greatly reducing a person’s ability to work (Kennedy and others 2003). Iron deficiency, the common cause of anemia, is associated with diminished work capacity and energy, leading to an estimated 1.5% loss in gross domestic product (Ramakrishnan 2002; Kennedy and others 2003). A deficiency in iodine is estimated to cause irreversible brain damage in 100,000 infants born to mothers who were deficient during pregnancy (Kennedy and others 2003). The effects of these three nutrients alone can have devastating effects on a person and community, making identification and treatment essential.

**Maternal malnutrition**

Malnutrition affects all age groups, but infants, young children and women, specifically pregnant and lactating women, are the most nutritionally vulnerable groups since they have physiologically higher nutrient requirements (Lartey 2008). In several countries in Sub-Saharan Africa, 10-19% of the female population in a given country is suffering from malnutrition, determined by a body mass index <18.5 kg/m² (Black and others 2008; Lartey 2008).

Malnutrition during pregnancy is critical as deficiencies can have short-and long-term consequences for both the mother and her child (Lartey 2008). The effects of maternal malnutrition are intergenerational, with malnutrition spanning at least three generations based on research that found an association between maternal height and the birth weight of their grandchildren (Victora and others 2008).
During pregnancy, a low BMI (<18.5 kg/m²), has been linked to intrauterine growth restriction, which is a risk factor for neonatal conditions including low birth weight and deficits in lean body mass in fetal composition (Black and others 2008; Larney 2008; Victora and others 2008). Malnutrition has also been shown to have long term effects on health through fetal programming, also known as the fetal origins hypothesis (Barker 1998). During fetal development, tissues and organs go through critical periods of growth where certain events, such as periods of malnutrition, can result in lifelong changes (Barker 1998). Research with animal models has shown that malnutrition during pregnancy leads to continuing changes in cholesterol metabolism, insulin response to glucose, blood pressure and immune function (Barker 1998).

**Causes of malnutrition**

Malnutrition is a serious condition with several underlying causes. The presence of civil unrest, or war, has been found to impact the number affected by malnutrition (O’Hare and Southall 2007; Bryce and others 2008). O’Hare and Southall (2007) conducted a study looking at 42 Sub-Saharan Africa countries, 21 of which had experienced recent conflict (1990-2004) and 21 countries that had not experienced recent conflict. Researchers found that there was an association between recent conflict and higher rates of malnutrition, maternal mortality and mortality in children less than five years of age (O’Hare and Southall 2007).

The burden of poverty is another cause increasing the number of people affected by malnutrition. Poverty limits an individual or family’s access to health services, nutritious foods, education and sanitary living conditions, increasing vulnerability to malnutrition (FAO: Food Security 2012; Kaseje and others 2005). Poverty creates a cycle as it compounds powerlessness, increasing ill health which in turn increases poverty (Kaseje and others 2005). Differences in the rates of malnutrition in a country are seen across a socioeconomic divide between poor and rich
populations (Van de Poel and others 2008). Cases of malnutrition have been found to cluster in poor communities within Sub-Saharan Africa (Van de Poel and others 2008). Research has shown that children from poor families are more likely to have less nutritious and diversified diets, report infectious diseases including fever and diarrhea, be exposed to unhealthy environments in the household, have reduced access to health services, and live in food insecure homes compared to children from wealthier families (Black and others 2008; Bryce and others 2008).

Limited access to healthcare and the expenses of treating malnutrition can result in a small number of people being treated when they need it most (Collins 2001; Hampshire and others 2009). Hampshire and others (2009) conducted field studies in Niger, where an estimated 63% of the 12.4 million in the country lived on less than a dollar a day in 2005. The researchers found that while caregivers did not neglect sick children, medical treatment at a clinic was not often utilized as the caregivers did not want to invest extra time and resources into just one child, especially if multiple visits were required. If the child was taken to a clinic, the caregivers’ still face opportunity costs including travelling long distances due to limited clinics and staying with children if they are admitted (Hampshire and others 2009). Admission into a clinic or hospital, especially in children, requires a caregiver to stay at the hospital until the patient is recovered (Collins 2001, 2004; Sadler and others 2007). It has been reported that on average, treatment at a hospital or clinic can take 27 days or longer, depending on the quality of care, which influences the food security and supply for the entire household since one caregiver is taken away from the home during this entire time (Sadler and others 2007).

Food insecurity in developing countries is a main factor causing the burden of malnutrition, especially when paired with the causes listed above. Food insecurity occurs when
people do not have physical and economic access to a safe, nutritious and sufficient food supply that meets the daily dietary needs of a person (Black and others 2008). Food insecurity is identified by the three main types: chronic, transitory and seasonal (FAO Food Security 2012). Chronic food insecurity occurs when people are unable to obtain the minimum nutrients needed over a long period of time while transitory food insecurity results from a sudden decline in the ability to access or produce enough food to meet the nutrition requirements over a short time period (FAO Food Security 2012). Seasonal food insecurity is associated with fluctuations in climate and cropping patterns that affect the amount of food available (FAO Food Security 2012).

While the underlying causes of malnutrition can be separated and identified from one another, they ultimately intertwine, creating a vicious cycle ending in a malnourished population. The presence of poverty results in the inability to gain access to nutritious foods leading to food insecurity resulting in hunger and malnutrition (FAO Food Security 2012). Malnutrition slows physical and cognitive development producing a population with low productivity and an inability to work (FAO Food Security 2012).

**Treatment of malnutrition**

While the exact treatment used for each patient varies depending on the severity of the case of malnutrition, a general treatment plan is recommended by the World Health Organization (WHO) (Walton and Allen 2011; WHO 2012). Moderate acute malnutrition is treated through the use of supplementary foods, also referred to as complementary foods, which provide the energy and other nutrients missing from the diet and are consumed in addition to the patient’s regular daily diet (Patel and others 2005; Manary and Sandige 2008). Supplementary foods
often consist of cereals and legumes fortified with micronutrients, often eaten as porridge, with a blend of corn and soy flour being the most commonly used (Patel and others 2005).

The traditional treatment used for a patient with SAM is split into two phases of treatments, as most have complications that require moderate amounts of nutrients to be introduced into the body first as patients often have poor appetite, impaired gut motility and decreased gastric volume (Manary and Sandige 2008). Complications commonly found in those with SAM include: a fever related to systemic infections, heart failure, respiratory distress, electrolyte imbalances, dehydration, hypoglycemia, hypothermia, marked anorexia, anemia, profuse diarrhea and shock (Manary and Sandige 2008; Walton and Allen 2011). The first phase in the treatment of complicated SAM involves hospitalization and feedings with small amounts of liquid food every two hours for the first day, followed by feedings every three to four hours until life threatening complications are under control (Ashworth and others 2003; Diop and others 2003; Ciliberto and others 2005; Manary and Sandige 2008; Walton and Allen 2011). The liquid diet, named F75, consists of milk powder, water, vegetable oil and sugar fortified with vitamins and minerals, with moderate levels of energy (75 kcal/100 mL) and protein (0.9 g/100 mL) (Diop and others 2003; Ciliberto and others 2005; Manary and Sandige 2008; Walton and Allen 2011). F75 contains reduced amounts of energy and protein to prevent exceeding the body’s metabolic capacity, as a protein intake greater than 1 g/kg body weight, along with impaired liver function and dehydration, results in the inability to remove excess ammonia (Müller and Krawinkel 2005). Once a patient’s appetite and clinical condition has improved, the second phase of treatment is started in which the patient is given a milk-based protein- and energy- dense diet, referred to as F100 (Ashworth and others 2003; Diop and others 2003; Ciliberto and others 2005; Manary and Sandige 2008; Walton and Allen 2011). The F100
formula contains 100 kcal/100 mL of energy and 2.9 g/100 mL of protein, which promotes rapid weight gain and is fed until a patient’s weight-for-height scores no longer suggest wasting (malnutrition) (Ashworth and others 2003; Diop and others 2003; Ciliberto and others 2005; Manary and Sandige 2008; Walton and Allen 2011).

Treatment with F75 and F100 requires hospitalization since both are nutrient rich liquids, which are excellent growth mediums for pathogenic bacteria (Diop and others 2003). The diets must be prepared in hygienic conditions with sterile drinking water and must be made before use each time since refrigeration is scarce, which prohibits home treatment (Briend and others 1999; Diop and others 2003; Briend and Collins 2010). In times of nutritional crisis, therapeutic feeding centers are set up solely for the treatment of malnutrition to reduce the hospital burden (Collins 2001, 2004). The intensive treatment plan for malnutrition requires high quality individual patient care, requiring a highly skilled, knowledgeable staff at the hospital or therapeutic feeding centers (Collins 2001, 2004; Collins and others 2006).

While hospitalization and treatment is successful in terms of recovery, the large demand for staff and infrastructure make this treatment option hard to sustain outside of emergency situations where aid is often provided (Briend and Collins 2010). Low numbers of properly trained health staff that has insufficient skills and motivation have been identified as a major limitation of this treatment option (Collins 2007). In Sub-Saharan Africa, 20 countries that are most affected by acute malnutrition have on average less than 4 doctors and 22 nurses per 100,000 population and these countries continue to experience critical shortages of trained and skilled health personnel (Collins 2007; Gupta and others 2011). In 2004, Mali had 4.4 doctors/100,000 and 12.6 nurses/100,000 which is below the recommended threshold of 23 doctors, nurses, and midwives per 10,000 (Collins 2007; Gupta and others 2011).
Once admitted to the hospital or therapeutic feeding center, a patient is often exposed to large numbers of other susceptible patients, permitting the spread of hospital acquired infections, increasing illness in those who are already very ill (Collins 2004, 2007; Walton and Allen 2011). Hospitalization of a child requires a caregiver to remain at the center until full recovery has been achieved resulting in high treatment costs and has a negative impact on family life for the other children, and weakens the economic and food security of the household as well (Collins 2001, 2004; Sadler and others 2007; Briend and Collins 2010; Walton and Allen 2011).

The recovery rates and the number of patients that stay nourished after treatment at a hospital or therapeutic feeding center is also of concern (Briend and Collins 2010). In 1987-1988 in a therapeutic feeding center in Tahou, Niger, 14.4% of admitted children died in the center, while 50% of children discharged with medical approval remained malnourished during follow-up examinations (Pécoul and others 1992). It has been reported that in Sub-Saharan Africa, only 25% of children treated at a hospital or therapeutic feeding center and discharged recover at home, approximately 10% die, 20% return to a center for additional treatment and 45% remain malnourished at home (Manary and others 2004).

**Ready-to-use therapeutic foods and community-based therapeutic care**

While the F75 and F100 treatment scheme does lead to successful recovery of patients, the total number reached is very low and limitations with this treatment plan led to a new model that included community involvement and the development and use of RUTFs (Collins 2004; Briend and Collins 2010). The foundation of the new model of care was that communities would be involved in identifying cases of malnutrition using symptoms and the MUAC measurement followed by home treatment using RUTFs (Briend and Collins 2010). Community-based therapeutic care (CTC) programs focus on the large number of severely malnourished people
within an area that can be treated through outpatient care instead of offering high quality care to a small portion of those who need it most (Collins 2004).

A critical component of CTC programs is having a treatment that can be used at home without becoming contaminated. RUTFs are energy dense foods that resist microbial growth and contamination that can be consumed directly without further preparation or the addition of water (Briend and others 1999; Briend 2001; Manary and others 2004; Briend and Collins 2010). The first RUTF was developed based on the F100 nutrient profile to provide a similar nutrient profile in a form that needed no additional preparation consisting of peanut butter, dried skim milk, oil, sugar, and the vitamin/mineral supplement (Briend and others 1999; Briend 2001). An early, small clinical trial using the RUTF was conducted in Mao, Chad used 20 children > 12 mo with SAM and the energy intake was 40.2 kcal/kg feed compared to 20.9 kcal/kg F100 (Briend and others 1999). This study provided preliminary results that the RUTF would be successful in the treatment of uncomplicated SAM, with further research needed (Briend and others 1999).

A larger clinical trial using the RUTF was conducted in Blantyre, Malawi with 282 HIV negative children >12 mo with SAM that had already been treated in the hospital for complications, including infections, and were released for phase two of treatment (Manary and others 2004). The children were assigned to 1 of 3 treatment groups: RUTF with 175 kcal/kg/day, RUTF supplement providing approximately 33% of the daily energy needed (~500 kcal/day) and a maize/soy flour diet. For the RUTF group, 95% of the children reached their goal weight, while only 78% of children receiving the RUTF supplement or flour blend reached their goal weight. All three home treatment options resulted in an 84% recovery rate overall, with weight and height gains being more rapid in the RUTF group (Manary and others 2004).
Another clinical trial focused on comparing the standard in-patient therapy to the home-based therapy using RUTFs in Malawi. A total of 1,178 children, aged 10-60 mo, were recruited for the study, with 186 receiving the in-patient therapy with F100 and 992 receiving home therapy with the RUTF (Ciliberto and others 2005). Of the 992 children treated with the RUTF, 65% did not require treatment at a hospital or therapeutic feeding center prior to receiving the RUTF. The results of the study showed that children treated at home had higher rates of recovery (weight for height z-score > -2) and relapsed less than children receiving the in-patient treatment. The children receiving the RUTF gained weight and height at a greater rate and had a higher MUAC compared to those receiving F100 with in-patient care. This study showed the efficacy of home based therapy with the RUTF in treating chronic malnutrition (Ciliberto and others 2005).

The results from these studies, along with other research with RUTFs, have shown that home based therapy with RUTFs is successful in treating moderate and severe acute malnutrition without complications (Manary and Sandige 2008; Briand and Collins 2010). People with complicated SAM require inpatient treatment to stabilize illnesses and can then be treated with RUTFs at home, reducing the length of hospitalization required (Manary and Sandige 2008; Briand and Collins 2010).

Research has also focused on producing the RUTF locally instead of importing the product from Nutriset, the manufacturer of the commercially available RUTF named PlumpyNut®, to reduce costs and make the therapy widely available to those who need it (Sandige and others 2004; Manary 2006). The RUTF can be made using local ingredients, except the powdered milk which is imported, and can be processed using a bakery mixer and filled into plastic containers (Sandige and others 2004; Manary 2006). Sandige and others
(2004) conducted a study in Malawi comparing locally produced RUTF to PlumpyNut® and the results showed that the local RUTF provided similar efficacy in the treatment of SAM.

**Plant-based RUTFs**

The success of PlumpyNut® and locally produced RUTFs using formulations similar to PlumpyNut® led to the United Nations recommending the use RUTFs at the community level for the treatment of severe acute malnutrition in children in 2007 (Dibari and others 2012). However, limitations with the current RUTFs do exist. Children under the age of five are the target group for most RUTFs, but they are not the only ones vulnerable to malnutrition. Breaking the cycle of malnutrition includes focusing on different target groups, specifically pregnant women as nutrition during pregnancy can have long lasting effects on both the mother and the infant (Briend and others 1999; Lartey 2008; Walton and Allen 2011; WHO 2012). Another drawback to the current RUTF formulations is that while the products are simple and can be produced locally, powdered milk must often be imported. Importing the powdered milk increases production costs and limits availability of the RUTFs to those who need it most (Dibari and others 2012).

While the most current RUTFs have limitations, new products can and are being developed that target different populations utilizing plant-based commodities to deliver the nutrition necessary at a reduced cost. Nabuuma and others (2012) developed a RUTF with a nutrition profile similar to F100 to be used as an alternative to F100, reducing the need for hospitalization. The RUTFs was produced using local ingredients of Uganda and contained a combination of peanuts, beans, cowpeas, amaranth grain and sugar (Nabuuma and others 2012).

More recently, plant-based RUTFs for adults have been developed and examined in research. Dibari and others (2012) have formulated a RUTF prototype for wasted children and
adults in East Africa using soybeans, corn, sorghum, oil and sugar that has a nutrient profile similar to PlumpyNut®. In a separate study, Bahwere and others (2009) used a chickpea-sesame based RUTF in the treatment of wasted adults (BMI < 17 kg/m²) with HIV. After 3 months of treatment, the researchers found that 73.3% of the patients had gained weight and had an increased BMI (Bahwere and others 2009). The results of this study suggest that plant-based ingredients can provide the nutrition needed for various target groups and lead to weight gain in a patient when blended in the right combinations and ratios.

Formulation of RUTFs

Linear programming

In the development of alternative RUTFs, a variety of techniques can be employed. One approach that has been suggested in developing RUTFs is linear programming. Linear programming is a technique that used to determine the best way to achieve the optimum outcome in a mathematical model based on a given list of requirements represented as linear equations (Al-Deseit 2009). Another way of defining linear programming is a computational method used for selecting, allocating and evaluating a limited number of resources based on linear algebraic constraints that will give an optimal solution for a problem (Udo and others 2011). The equations used in linear programming were first developed by G.B. Dantzig in 1947 to solve problems relating to World War II. Today, linear programming is widely used in all fields to find optimal solutions to a range of problems (Al-Desiet 2009; Winfeed 2012). The equations displayed below show a basic linear programming model with the goal being to minimize the objective function, often representing the cost of a formulation, with food ingredients and needed nutrients being the variables:

\[
\text{Minimize } \sum_{j=1}^{n} c_j x_j (j=1, 2, 3, \ldots, n)
\]
subject to \( \sum_{j=1}^{n} a_{ij}x_j \leq b_i \) for \( i = 1, 2, \ldots, m \)

\[
\sum_{j=1}^{n} a_{ij}x_j \geq b_i \text{ for } i = 1, 2, \ldots, m
\]

\[
\sum_{j=1}^{n} x_j = 1
\]

\( x_j \geq 0 \)

where:

- \( x_j \): the number of units of the raw food ingredient \( j \) in the solutions;
- \( c_j \): the cost of the raw food ingredient \( j \);
- \( a_{ij} \): the quantity of the nutrients \( i \) in one unit of the raw food ingredient \( j \);
- \( b_i \): the specific number of units of nutrient \( i \) required;
- \( m \): the number of nutrients or rows;
- \( n \): the specific number of raw food ingredients or columns;

(Dantzig 1951, 1955; Church and others 1963; Anderson and Earle 1983; Winfeed 2012).

Linear programming does have limitations like all mathematical models that must be known and understood. An important assumption in linear programming is that one ingredient is a linear substitution for any other ingredient according to their specified relative nutritional composition (Church and others 1963). The model also assumes that the nutritional content of an ingredient can be specified without any error; however, nutrient composition depends on the growing conditions in a given region (Ge and others 2010). The quality of developed formulations depends on the information available with inadequate information, nutrient variability within an ingredient, possible interactions between ingredients and difficulty in
predicting taste of a formulation all influencing the results and amount of error seen in a formulation (Church and others 1963).

Despite limitations, linear programming is extensively used in the animal industry to develop least-cost feed formulations and is utilized in most commercially available feed formulation software programs (Udo and others 2011). Tozer (2000) used linear programming to develop a series of feed rations for dairy heifers at different stages of growth and found that the ingredients across each model were relatively similar, with dry shelled corn and soybean meal varying in the formulations to meet the different nutrient constraints. Udo and others (2011) used Pearson’s square and linear programming models to develop rations for fish in an aquaculture system in Nigeria using local ingredients. The researchers found that the feed ration developed with linear programming incorporated the lowest cost ingredients more efficiently than the Pearson’s square model since the linear programming took the price of each ingredient into account (Udo and others 2011). Of the two formulations developed, the ration designed with linear programming resulted in catfish with a higher final weight and a higher rate of gain compared to those fed the ration developed using the Pearson’s square model (Udo and others 2011).

**Linear programming in human diet planning**

Linear programming has also been used to plan human diets from an individual level to a national and global level based on foods available in a given area (Anderson and Earle 1983). Smith (1959) first used linear programming to develop model diets for humans that meet nutritional and conventional requirements at the least-cost possible for a family of three for four weeks. Using this model, Smith (1959) developed three diets with different nutrient constraints based on foods frequently purchased by families in Lansing, Michigan in 1955. Darmon and
others (2002) used a linear programming to plan a least-cost diet for pre-school children in Malawi using locally available ingredients. The results of the study by Darmon and others (2002) suggest that linear programming can be utilized in the development of adequate diets.

While linear programming was first used to develop diets for humans, this model has since been used in formulating least-cost, nutritious food products. Valencia and others (1988) developed an infant food using only plant ingredients consisting of chickpeas, rice, soybean meal and bananas that was processed into a dry cereal. In another study, El-Habashy and others (1995) used linear programming to formulate a weanling food based on local ingredients available in Egypt, along with NFDM, with the results showing that the nutrient content predicted was similar to the actual values obtained through analysis. Mensa-Wilmot and others (2003) designed weaning food supplements using local Ghanian cereals, pulses and oilseeds that were determined to meet at least one-third of the daily requirements of the target nutrients necessary for weaning infants.

The use of linear programming in formulating foods for human consumption has been found to be a useful tool in developing nutrient-dense foods using available ingredients. However, as with the RUTFs currently available, most foods that have been developed with linear programming only target the nutrient requirements of infants and children. Little research has focused on using linear programming to develop nutrient-dense RUTFs for malnourished pregnant women. Linear programming coupled with plant-based local ingredients from a specific area would allow RUTFs to be tailored to a target population in a specific region.

**Region of focus**

Many areas worldwide are affected by malnutrition, however, West Africa, is one of the least developed regions in the world (Atlas West-Africa 2012). West Africa, a sub-region of
Africa, is comprised of 17 countries covering the section of Africa that is bordered by the Atlantic Ocean to the west and south and by the Saharan Desert to the north (FAOa 2011; Atlas West-Africa 2012). The 17 countries of West Africa include: Benin, Burkina Faso, Cape Verde, Chad, Côte d’Ivoire, the Gambia, Ghana, Guinea, Guinea-Bissau, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone and Togo (FAOa 2011), which are marked and shown in Figure 2.1. The lack of development in this region is the result of several structural factors: poverty, political instability and conflicts, civil wars, weak economies dependent on international markets, high population growth, low education levels and limited access to basic services (FAOa 2011; Atlas West-Africa 2012).

Agriculture plays a key role in the economies of West Africa, with more than half of the rural population depending directly on rural crops. The major crops grown in this region include millet, sorghum, maize, plantains, rice, roots and tubers (FAOa 2011). Food security is a major problem in West Africa, especially for those whose main food supply is the crops they produce, as natural disasters, climate change and plant diseases threaten food production and lead to increased child malnutrition (FAOa 2011). Adult malnutrition is also a problem in West Africa as 7-10% of women in the coastal countries and 13-20% of women in the Sahelian area aged 15-49 years have a BMI <18.5 kg/m$^2$, indicating a chronic energy deficiency (Lopriore and Muehloff 2003).

**Country of Focus**

Narrowing the focus within West Africa, Mali is a landlocked country that has a young population with a life expectancy at birth of 53 years that is devastated by climate and civil wars (CIA World Factbook 2012) (marked in Figure 2.1). Mali has a total population of 15,494,466, with 0-14 year olds comprising 47.8% of the population, 15-64 year olds accounting for 49.2%
of the population and those ≥65 years of making up 3% of the population (CIA World Factbook 2012; Nutrition and Consumer Protection 2012). Along with a young population, Mali has a maternal mortality ratio of 540 deaths/per 100,000 live births, the 16th highest in the world, with the maternal mortality ratio accounting for the number of females who die from a cause related to or aggravated by pregnancy (CIA World Factbook 2012).

Mali, being strongly affected by climate, has three main climate zones with varying amounts of rainfall: arid (<200 mm rain), Sahelian (200-600 mm rain) and Sudanian (800-1200 mm rain), which influence the crops that can be grown (FAOa 2011; CIA World Factbook 2012). The diet of Malians is based mainly on cereals, providing two-thirds of dietary energy, that include rice, millet, sorghum and maize, along with pulses (mainly cowpeas), fruits and vegetables and starchy roots such as sweet potatoes, yams and cassava (Nutrition and Consumer Protection 2012). Other crops consumed include fonio, groundnuts and sesame (Torheim and others 2004; Aly and others 2011). This carbohydrate based diet, along with chronic food insecurity and limited access to health care, has resulted in high rates of malnutrition. In children less than five years old in 2006, 15% were wasted and 38% were stunted, placing Mali at a high level of malnutrition (Nutrition and Consumer Protection 2012).

Previous and current conflict in Mali has also lead to food insecurity and high levels of malnutrition. Ongoing conflict in northern Mali has put an estimated 4.6 million people at risk of food insecurity, with an estimated 560,000 children under the age of five at risk of malnutrition. In August of 2012 alone, 16,334 children and 2,329 pregnant/lactating women were enrolled in treatment programs for moderate acute malnutrition (OCHA 2012).
Figure 2.1. Map of Africa with the region of West Africa outlined in the dark black line. Mali, the country of focus for the present work, is circled in black and labeled with white text (reprinted with permission; adapted from Black and others 2008).
Selected ingredients

Current RUTFs contain two main ingredients, peanuts, which can be grown locally and powdered milk that must be imported, increasing the total cost (Manary 2006). One option to reduce cost and increase access to RUTFs is to use locally available plant ingredients (crops) in a given region or country (Nout and Ngoddy 1997). The plant ingredients used must provide adequate nutrition and a complete amino acid profile; therefore it is important to include cereals, legumes and oilseeds (Mensa-Wilmot and others 2003). In Sub-Saharan Africa, cereals are the main source of calories in the diet and are a good source of B-complex vitamins, methionine and cysteine but are limiting in lysine. Legumes are a good source of lysine but are limiting in methionine and cysteine. Combining cereals with legumes in a 50:50 ratio results in a complete protein source since cereals and legumes are complementary protein sources (Mensa-Wilmot and others 2003). The eleven ingredients selected for the RUTFs in the present work based on locally available ingredients in Mali are described below. The nutrient composition for all ingredients is shown in Table 2.1.

Rice (*Oryza sativa* L.) is a staple cereal for over half of the world’s population, including developing countries in Africa. The two main species of rice cultivated are *O. sativa* (Asian rice) and *O. glaberrima* Steud (African rice) (Juliano 1972). Rice has a protein content between 5-7%, which is lower than the protein content found in most cereals. However, rice proteins have a lysine content that is more than 50% greater than wheat and has a good amino acid balance (Friedman 1996). The carbohydrate content of rice is approximately 80%, which is the source of energy from rice (USDA 2012).

Corn (*Zea mays* L.), also known as maize, is a tall annual plant belonging to the grass family and is a warm season crop that requires abundant moisture (Benson and Pearce 1987;
Purdue University 1999). Corn, rice and wheat are the three cereals whose crop production greatly exceeds those of other crops and are major staples in the human diet throughout the world (Benson and Pearce 1987). A basic cereal staple for many people in Africa, Latin America and parts of Asia, corn contributes more than half of a person’s daily calorie and protein intake levels (Friedman 1996). As a food source with 9% protein, 72% starch and high in leucine, corn is readily digested by humans and is one of the best metabolizable energy sources among the various grains (Wright 1987; Hoseney 1994; USDA 2012).

Sorghum (Sorghum bicolor (L) Moench) is a tropical grass grown in semi-arid parts of the world, including large acres in Africa and Asia where the climate is often too hot and dry for corn (Carter and others 1989; House 1995). The major growing area for sorghum in Africa runs across the West African region south of the Saharan desert almost to the coast and then eastward into Sudan, Ethiopia and Somolia (House 1995). Worldwide, sorghum is used as a food cereal grain for humans, providing more protein than corn (11% vs 9%) and a starch content of 75% (Carter and others 1989; USDA 2012). Corn and sorghum have similar chemical compositions, but sorghum has been shown to be less efficient in lab animal and livestock feeding studies. Protein digestibility studies in rats have shown that intermediate textured sorghum endosperm was found to have a digestibility of 70.3% compared to 78.5% for corn (Klopfenstein and Hoseney 1995).

Pearl millet (Pennisetum glaucum (L) R. Br.) originated in the African savannah south of the Saharan desert and west of the Nile river and has been grown since prehistoric times (Oelke and others 1990; House 1995). Today, pearl millet is still grown as a food grain, with the highest producing countries including Nigeria, Niger, Mali, Chad, Tanzania, Ethiopia, China and India (House 1995). As a nutrient source, pearl millet has a starch content of 73%, a protein content of
approximately 11% and a high leucine content (Hoseney 1994; Klopfenstein and Hoseney 1995; USDA 2012). Pearl millet has a true protein digestibility of 94-97% based on rat studies and has been found to have a higher protein and calorie digestibility than wheat flour (Klopfenstein and Hoseney 1995).

Barley (*Hordeum vulgare* ssp.) is one of the most ancient grains still cultivated today and is the fourth most commonly used grain worldwide (Friedman 1996; Purdue University 1999). While the primary use of barley is for beer production, this cereal is also used for as a human food source (Friedman 1996; Purdue University 1999). When used for human consumption, the barley is pearled by using abrasive disks to grind the hull and bran from the kernels (Purdue University 1999). The composition of barley is approximately 78% starch and 10% protein (USDA 2012). While all cereals are limiting in lysine, barley has a higher lysine content than most, with 0.37 g/100 g of barley compared to 0.25 g/100 g of lysine in corn (Hoseney 1994; USDA 2012).

Fonio (*Digitaria exilis* Stapf), also known as acha or hungry rice, is a grain indigenous to the savannah regions of West Africa. Fonio is not produced on a large scale for human consumption due to the tedious nature of the post-harvest processing of the grain, including the polishing of the small grains (Clottey and others 2006). The protein content of fonio is similar to other cereals, ranging from 8.7-11.8% depending on the species grown, with a high methionine (4.8%) and cysteine content (2.5%) (de Lumen and others 1993; Friedman 1996; Clottey and others 2006).

Cassava (*Manihot esculenta*), also known as manioc or yucca, is a drought tolerant plant that is a staple food found in the tropical and subtropical regions of Africa, Asia and Latin America (Montagnac and others 2009). Cassava serves as a major carbohydrate (energy) source
for approximately 500 million people. The exact nutritional content of cassava depends on the portion of the plant eaten, the root or the leaves, with the root being the energy dense part of the plant. The cassava root has a carbohydrate content making up 32-35% of the root on a fresh weight basis (80-90% dry basis) and protein content of 1-3% of the root dry basis (Montagnac and others 2009). However, cassava does have anti-nutritional properties and toxic substances that limit its consumption. Cassava root has a cyanide content ranging from 10-500 mg cyanide equivalents/ kg dry matter, which is higher than the Food and Agriculture Organization (FAO)/WHO recommendations of consuming <10 mg cyanide equivalents/ kg dry matter to prevent acute toxicity. Before consumption, cassava root must be processed to remove the toxic cyanogens (Montagnac and others 2009).

Yams (*Dioscorea* spp.) are herbaceous plants with a twining stem that produce edible starchy storage tubers (Agbor-Egbe and Treche 1995). This tuber is widely grown in West Africa and is an important source of calories, carbohydrates and other nutrients in the tropics (Wanasundera and Ravindran 1994; Afoakwa and Sefa-Dedeh 2001). Yams have an average starch content ranging from 70.4 to 80% and a crude protein content ranging from 5.2-9.6%, depending on the species grown, which is higher than the protein content of other root crops such as cassava, taro and sweet potato (Wanasundera and Ravindran 1994; Agbor-Egbe and Treche 1995).

Sesame (*Sesamum indicum* L.), one of the oldest cultivated plants in the world, is largely grown in India and China followed by Burma, Sudan, Mexico, Nigeria, Venezuela, Turkey, Uganda and Ethiopia (Oplinger 1990). Sesame is a rich source of fat, with a content of approximately 50%, and a protein content of 17% (Oplinger 1990; USDA 2012). A large
percentage of the fats found in sesame are unsaturated, specifically oleic (47%) and linoleic (39%) (Oplinger 1990).

Cowpeas (*Vigna unguiculata* L. Walp.), also referred to as blackeye peas, southern peas or crowder peas, are a legume that originated in Africa and were thought to have been domesticated with sorghum and pearl millet because of the close association of these crops in early African farming (Ng and Maréchal 1985; Davis and others 1990). These legumes are a good source of protein with a content of approximately 25%. Cowpeas are rich in lysine and tryptophan but are low in the sulfur containing amino acids, methionine and cysteine (Davis and others 1990; USDA 2012).

Peanuts (*Arachis hypogea* L.) are energy dense, nutritious food that originated in South America and are now grown throughout the world in tropical and warm temperate regions. The growing regions for peanuts include several African countries, India, the United States, China and Argentina (Putnam and others 1991; Higgs 2003). Peanuts are an important oilseed and a legume that serves as a food source, especially in developing countries, with a protein content ranging from 25-32%. The fat content of peanuts ranges from 42-52%, with at least 75% of the fat being unsaturated; nearly half of the unsaturated fats are monounsaturated oleic acids (Putnam and others 1991; Higgs 2003; USDA 2012).
### Table 2.1 Nutrient composition of the eleven local plant ingredients in Mali for RUTF formulation based on nutrient profiles from the USDA (2012) Nutrient Database

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Peanuts</th>
<th>Cowpeas</th>
<th>Sesame</th>
<th>Rice</th>
<th>Yellow</th>
<th>Corn</th>
<th>Sorghum</th>
<th>Pearl</th>
<th>Fonio</th>
<th>Cassava</th>
<th>Barley</th>
<th>Yam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>25.80</td>
<td>23.52</td>
<td>17.73</td>
<td>6.61</td>
<td>9.42</td>
<td>11.30</td>
<td>11.02</td>
<td>9.33</td>
<td>1.36</td>
<td>9.91</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>Fat (%)</td>
<td>49.24</td>
<td>1.26</td>
<td>49.67</td>
<td>0.58</td>
<td>4.74</td>
<td>3.30</td>
<td>4.22</td>
<td>1.94</td>
<td>0.28</td>
<td>1.16</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Carbohydrates (%)</td>
<td>16.13</td>
<td>60.03</td>
<td>23.45</td>
<td>79.34</td>
<td>74.26</td>
<td>74.63</td>
<td>72.85</td>
<td>73.57</td>
<td>38.06</td>
<td>77.72</td>
<td>27.88</td>
<td></td>
</tr>
<tr>
<td>Ash (%)</td>
<td>2.33</td>
<td>3.24</td>
<td>4.45</td>
<td>0.58</td>
<td>1.20</td>
<td>1.57</td>
<td>3.25</td>
<td>3.17</td>
<td>0.62</td>
<td>1.11</td>
<td>0.82</td>
<td></td>
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<tr>
<td>Fiber (%)</td>
<td>8.50</td>
<td>10.60</td>
<td>11.80</td>
<td>-</td>
<td>7.30</td>
<td>6.30</td>
<td>8.50</td>
<td>-</td>
<td>1.80</td>
<td>15.60</td>
<td>4.10</td>
<td></td>
</tr>
<tr>
<td>Energy (kcal/100 g)</td>
<td>567.00</td>
<td>336.00</td>
<td>573.00</td>
<td>360.00</td>
<td>365.00</td>
<td>339.00</td>
<td>378.00</td>
<td>351.94</td>
<td>160.00</td>
<td>352.00</td>
<td>118.00</td>
<td></td>
</tr>
</tbody>
</table>

Indispensable Amino Acids (%)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Peanuts</th>
<th>Cowpeas</th>
<th>Sesame</th>
<th>Rice</th>
<th>Yellow</th>
<th>Corn</th>
<th>Sorghum</th>
<th>Pearl</th>
<th>Fonio</th>
<th>Cassava</th>
<th>Barley</th>
<th>Yam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>0.25</td>
<td>0.29</td>
<td>0.39</td>
<td>0.08</td>
<td>0.07</td>
<td>0.12</td>
<td>0.12</td>
<td>0.09</td>
<td>0.02</td>
<td>0.17</td>
<td>0.01</td>
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</tr>
<tr>
<td>Threonine</td>
<td>0.88</td>
<td>0.90</td>
<td>0.74</td>
<td>0.24</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>0.18</td>
<td>0.03</td>
<td>0.34</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.91</td>
<td>0.96</td>
<td>0.76</td>
<td>0.29</td>
<td>0.34</td>
<td>0.43</td>
<td>0.47</td>
<td>0.13</td>
<td>0.03</td>
<td>0.36</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>1.67</td>
<td>1.80</td>
<td>1.36</td>
<td>0.55</td>
<td>1.16</td>
<td>1.49</td>
<td>1.40</td>
<td>0.41</td>
<td>0.04</td>
<td>0.67</td>
<td>0.10</td>
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<tr>
<td>Lysine</td>
<td>0.93</td>
<td>1.59</td>
<td>0.57</td>
<td>0.24</td>
<td>0.27</td>
<td>0.23</td>
<td>0.21</td>
<td>0.18</td>
<td>0.04</td>
<td>0.37</td>
<td>0.06</td>
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<tr>
<td>Methionine</td>
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<td>0.34</td>
<td>0.59</td>
<td>0.16</td>
<td>0.20</td>
<td>0.17</td>
<td>0.22</td>
<td>0.28</td>
<td>0.01</td>
<td>0.19</td>
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<td>0.14</td>
<td>0.17</td>
<td>0.13</td>
<td>0.13</td>
<td>0.29</td>
<td>0.03</td>
<td>0.22</td>
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</tr>
<tr>
<td>Phenylalanine</td>
<td>1.34</td>
<td>1.37</td>
<td>0.94</td>
<td>0.35</td>
<td>0.46</td>
<td>0.55</td>
<td>0.58</td>
<td>0.22</td>
<td>0.03</td>
<td>0.56</td>
<td>0.07</td>
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<tr>
<td>Tyrosine</td>
<td>1.05</td>
<td>0.76</td>
<td>0.74</td>
<td>0.22</td>
<td>0.38</td>
<td>0.32</td>
<td>0.34</td>
<td>0.08</td>
<td>0.02</td>
<td>0.28</td>
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<tr>
<td>Valine</td>
<td>1.08</td>
<td>1.12</td>
<td>0.99</td>
<td>0.40</td>
<td>0.48</td>
<td>0.56</td>
<td>0.58</td>
<td>0.22</td>
<td>0.04</td>
<td>0.49</td>
<td>0.06</td>
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<tr>
<td>Histidine</td>
<td>0.65</td>
<td>0.73</td>
<td>0.52</td>
<td>0.16</td>
<td>0.29</td>
<td>0.25</td>
<td>0.24</td>
<td>0.12</td>
<td>0.02</td>
<td>0.22</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>All nutrient profiles were found in the USDA (2012) Nutrient Database, except for Fonio which was found in the literature (Clottey and others 2006).
Processing of Ready-to-Use Therapeutic Foods

Along with ingredient selection, the processing techniques used in the production of RUTF must be considered. Processing techniques are employed to improve the bioavailability and nutritional content of raw plant ingredients. In the developing world, simple techniques used in the household setting can be identified and utilized to process nutrient-dense foods. Processing methods often used include roasting, thermal methods such as boiling, dehulling, pounding, soaking, germination, enzymatic hydrolysis and fermentation (Nout and Ngoddy 1997; Hotz and Gibson 2007). In Mali, anthropological work has found that in the home setting wet cooking techniques such as steaming, boiling/stewing and frying are used to prepare almost every cooked dish. Market vendors and bakeries in Mali use a combination of dry methods including roasting, grilling and baking to prepare breads, cakes and various meats (MacLean and Insoll 1999).

Each processing method serves a specific function in the development of food products. Some processing methods are used to improve the nutritional content of a food by reducing anti-nutritional factors. Plant-based foods often contain protease inhibitors and anti-nutritional compounds, such as phytate, that are often found in corn, rice and sorghum. Tubers, like cassava, must be processed before consumption to reduce the levels of poisonous phytotoxins such as cyanogenic glycosides (Nout and Ngoddy 1997; Hotz and Gibson 2007).

A combination of processing techniques is often necessary to produce an optimal food product, especially when using the raw plant ingredients available in developing countries. Nout and Ngoddy (1997) developed a basic framework for optimizing the formulation and processing of weaning foods for infants made from locally available plants. The first stage of the process, referred to as the preparative step involved a sequence of methods, such as detoxifying, dehulling
and pounding, to convert the raw materials into useable forms of ingredients that can be blended together. The second stage of the processing scheme brought individual ingredients together in the proper amounts to create a mixture for final processing. Stage three of the process combined unit operations, such as grinding, enzymatic hydrolysis, thermal treatments and packaging to create the final product (Nout and Ngoddy 1997). This basic processing scheme is the foundation of any food processing operation, however, the framework described above was designed to specifically account for the utilization of multiple simple technologies to transform the various local plant crops into a nutrient dense RUTF.

**Enzymatic hydrolysis in food processing**

Enzymatic hydrolysis is one simple technology method that has been used for centuries in food processing to modify nutritional and physical characteristics of food products. Enzyme technology has evolved from basic forms of malt, koji, calf stomachs and papaya leaves previously to the purified enzyme sources used today (Adler-Nissen 1987). Enzymes are utilized in the food industry to break down components such as starch and protein, improving their availability, while also altering the viscosity of the product (Rolle 1998). Today, enzymes are used in several sectors of the food industry including brewing, baking, dairy, fruit and wine and distilling to enhance the final product (Adler-Nissen 1987; Arbige and Pitcher 1989).

The primary sources of enzymes used in the industry are plants, animals and microorganisms (Adler-Nissen 1987; Rolle 1998). Microbial enzymes are produced commercially through submerged solid substrate fermentation. Submerged fermentation for enzyme production is conducted in stirred tank reactors under aerobic conditions using batch or fed-batch systems. Solid substrate fermentation involves microbial growth on or within particles of a solid substrate under aerobic conditions with little to no free water. In solid substrate
fermentation, the crude fermented product produced can then be used directly as the enzyme source, with no additional preparation (Pandey and others 2000). Considering the two enzyme production methods for use in developing countries, submerged fermentation requires a high capital investment and has high energy and infrastructural needs for large-scale enzyme production (Rolle 1998). Solid substrate fermentation, however, is a low cost, low energy simple fermentation process that can be used for small scale production of enzymes, especially in developing countries (Rolle 1998; Krishna 2005). The cost of producing cellulase by submerged and solid substrate fermentation was compared and determined that through submerged fermentation, cellulase production was $20/kg while in solid substrate fermentation the price was $0.2/kg (Pandey and others 2000).

Koji, a type of solid substrate fermentation, is an ancient technique thought to have originated 3,000-2,000 years ago and migrated to Japan sometime between BC 10th-3rd AD century (Krishna 2005; Machida and others 2008). This fermented product has been used as a starter in the soy sauce industry, the fermentation of miso, brewing of sake (rice wine) and several other oriental foods (Krishna 2005). Preparation of koji begins with growing mold, usually *Aspergillus oryzae*, on steamed or cooked rice, soybeans, wheat bran or mixtures of these commodities. *A. oryzae* serves as one of the main sources of hydrolytic enzymes, such as amylases, proteases and lipases, in koji to hydrolyze the substrate during fermentation (Chou and Rwan 1995). Fungal amylases and proteases, such as those produced by *A. oryzae*, have several applications in which they can be utilized in the food industry.

Production of amylases and proteases through fermentation with *A. oryzae* (koji) has been researched and reported. Chutmanop and others (2008) found a peak α-amylase activity of 8,000 units of enzyme per g of dry solids and a peak protease activity of 850 units during
fermentation at 12 and 90-100 h, respectively. In a separate study, Sivaramakrishnan and others (2007) reported an α-amylase activity of 9,065 units of activity per g of dry solids. Chou and Rwan (1995) studied the acid and neutral proteases produced by *A. oryzae* and found activities of 1,600 and 1,400 units per g of dry matter. The α-amylase and protease activities found in these studies described above are comparable suggesting koji is a rich source of α-amylases and proteases.

Amylases from *A. oryzae* are more efficient in the saccharification of starch compared to bacterial α-amylases. These α-amylases produced by *A. oryzae* and have been utilized in food industry applications including anti-staling in the baking industry, clarification of haze in fruit juices and alcoholic beverages and the production of glucose and maltose syrup production (Sivaramakrishnan and others 2007). Alpha amylases degrade starch by randomly cleaving the 1,4-α-D-glucosidic linkages between the glucose units in linear amylose chains (Kammoun and others 2008). The maximum level of starch degradation by α-amylases is achieved when food processing procedures are used to destroy the starch structure, releasing component molecules that are available for enzymatic hydrolysis (Oates 1997). In general, thermal treatment in an aqueous environment is the principle process through which starch becomes available for enzymatic degradation (Singh and others 2010).

Hydrolysis of proteins in food products through the use of proteases can affect the functional properties of proteins including solubility, water holding capacity, gelation, emulsification and foaming properties (Panyam and Kilara 1996). Proteases are one of the most commercially important enzymes, which account for 60% of the industrial enzyme market and used in industries including pharmaceutical processing, leather making, detergents and food processing (Krishna 2005; Wang and others 2005; Chutmanop and others 2008).
Proteases hydrolyze proteins by splitting peptide linkages between amino acids, producing a mixture of peptides with different molecular sizes and free amino acids (Clemente 2000). Industrial proteases are classified on both their origin and their specific catalytic mechanism, either exopeptidases or endopeptidases. Exopeptidases are responsible for cleaving the peptide bond that is proximal to the amino or carboxy termini of the substrate. Endopeptidases, however, split peptide bonds that are distant from the termini of the substrate (García-Carreño 1991; Rao and others 1998; Clemente 2000).

Plants represent one source of proteases that can be easily obtained in developing countries. One example is bromelain, a crude, aqueous extract from the stems and immature fruits of pineapples. Bromelain is a complex mixture of thiol-endopeptidases that have enzymatic activities over a wide pH range, with an optimum pH between 5.5-8.0 and an inactivation temperature of 70 °C (Rao and others 1998; Maurer 2001).

The inability, however, of plant and animal proteases to maintain current demands has led to increased interest in microbial proteases, from sources such as A. oryzae, with microbial enzymes accounting for approximately 40% of all protease sales (Rao and others 1998). The use of proteolytic enzymes in the food industry increased with the development of fermentation techniques the produced high yields of proteases at a low cost (García-Carreño 1991).

**Thermal processing**

Enzymatic hydrolysis is used to alter the physical and nutritional properties of food products, but other processes are required to produce high quality, safe, shelf-stable foods. Thermal processing has a long history in food processing of leading to food products that have a long shelf life (Durance 1997). The main purpose of thermal processing is to destroy pathogenic and spoilage microorganisms, inactivate enzyme systems that lead to food degradation and
destroy heat-labile toxins (Leonard and others 1986). Three forms of thermal processing used in the food industry today include pasteurization, sterilization and aseptic processing (Cutter 2002). Pasteurization employs a mild heat treatment for a short amount of time that is aimed at inactivating enzymes and destroying most vegetative bacterial cells to enhance safety and extend shelf life of a product, like ready-to-eat chilled meals that have a shelf life of approximately 10 days. Commercial sterilization, also known as canning, is the preservation of foods in hermetically sealed containers that results in a product that is free of pathogenic bacteria and has a long shelf-life, being microbiologically stable for up to two years (Cutter 2002; Gaze 2005). In commercial sterilization, the food product is filled into cans or jars, sealed and then processed to produce a safe, shelf-stable product (Cutter 2002). The third type of processing method, aseptic processing, involves filling a sterile food product into pre-sterilized containers in a sterile room (Cutter 2002). This thermal process requires the greatest amount of control since there are several steps where contamination may occur and no final processing step like commercial sterilization that will kill all vegetative cells (Cutter 2002).

Shelf-stable food products that do not require refrigeration are a necessity in developing countries where refrigeration is not readily available to most households (Leistner 1992). Of the three thermal processes described above, commercial sterilization is the best option for processing a safe, shelf stable product in developing countries that does not require refrigeration afterwards (pasteurization) and does not require a sterile processing environment (aseptic).

Commercial sterilization of food products is often achieved through the use of a retort. The two main types of retorts used are still retorts where the food product remains stationary or rotary retorts which agitate the product during processing (Ávila and others 1999). The exact sterilization process used in producing each food product is different based on the characteristics
of each product and the type of retort used. The time needed for sterilization also varies depending on the product, the container material and size, the orientation of the packages and the characteristics of the heating medium (Ghani and others 1999).

While thermal sterilization results in a shelf-stable safe product, the process does have negative effects on the food product. The heat required to destroy the microbial pathogens, spoilage microorganisms and the endogenous and introduced enzymes also destroys heat-labile vitamins such as vitamin C, thiamin and folic acid. The quality of food products is also degraded, with properties such as color, flavor and texture being affected (Durance 1997).

**Microbial safety in food processing**

Thermal sterilization requires balancing the loss of quality and nutritional properties with the safety of the food product that is provided (Durance 1997). Providing a safe product is especially important in malnourished patients who are already weak and susceptible to infections. Considering thermal sterilization from a microbial standpoint, a key parameter is the target organism, either pathogenic or spoilage, that must be adequately reduced during processing (Gaze 2005). The quality of the raw ingredients used in the food product is an important factor microbiologically as the initial microbial load of the raw ingredients, including the different types of microbes and the concentration, influences the processing time. The heating process used must reduce the initial level of microorganisms found in the ingredients to an acceptable level in the final product (Gaze 2005).

Product characteristics play a role in the microbial safety of the product and influence the final thermal process. Viscosity of the carrier fluid affects heat penetration into the product, with thicker products, like concentrated starch, having a slower rate of penetration. A slow rate of heat penetration requires a longer heat treatment to achieve the same microbial destruction as a
thinner product. A nutritious product high in fats, protein, and/or total solids can also present problems, as the nutrients increase the heat resistance of the contaminating microorganisms by a factor of two to three when compared to the standard resistance of a given microorganism in broth (Gaze 2005). When these microbial concerns are taken into consideration, a thermal sterilization process must be developed with the appropriate time and temperature that will result in a safe, commercially sterile food product.

Packaging in product stability and safety

The processing method used to provide a safe product to the consumer is an important factor, but the type of package used to deliver the product also plays a key role in the shelf life of a food product. Packaging of food products serves four main functions: protection, containment, providing information to the consumer and utility of use (Cutter 2002). In general, packaging provides protection from chemical, biological and physical influences that can affect the food within. Chemical influences such as exposure to gases, light and water trigger compositional changes in the food that are undesirable. Biological exposures, microorganisms, insects, rodents and other animals can contaminate a food product if not properly protected. Physical impacts, such as mechanical damage, vibrations and shock during distribution and transport can damage and destroy a food product (Marsh and Bugusu 2007). The packaging materials used for a food product must also protect against these influences to deliver a safe, high quality final product to consumers.

The materials used in food packaging has evolved from tree trunks, gourds and animal hides to the materials commonly used today which include glass, metal, plastics, paper and paperboard (Cutter 2002; Marsh and Bugusu 2007). The use of glass in food packaging has a long history dating back to 3,000 B.C. when Egyptians used pottery and blown glass to protect
their food (Cutter 2002; Marsh and Bugusu 2007). Glass is an excellent packaging material since it is odorless and chemically inert with almost all foods. Packages made from glass are also impermeable to gases and vapors, maintaining the freshness of foods without impairing taste. Another advantage of glass is that it is a rigid packaging material that can be produced in many shapes while still providing protection and good insulation and withstanding the high processing temperatures encountered during thermal sterilization. The main disadvantages of glass packages are that they are heavy, brittle and susceptible to breakage from internal pressure, impact and thermal shock (Marsh and Bugusu 2007).

Metal, another commonly used packaging material, is often shaped into cans made of aluminum, tinplate steel or combinations of both materials to provide the strength, protection and barrier properties characteristic of metal containers. Packages made of metal are primarily used for hot fill and processed foods due to the metal’s speed of manufacture, ease of filling, ease of closing and ability to withstand high processing temperatures and pressure. The main disadvantages of metal packages, however, are the weight of the material and the inability to reclose or reuse the container (Cutter 2002).

The use of plastic packaging materials has become more prevalent as it provides a cheaper option while still providing protection for the food product. The type of plastic used to make the rigid containers can be selected specifically based on food compatibility, the type of processing method used and the storage needs of the product (Cutter 2002). However, the barrier quality of plastic containers is lower and they also have a lower resistance to heat and pressure (Cutter 2002; Marsh and Bugusu 2007). Paper and paperboard are often used in corrugated shipping boxes, milk cartons, bags and folding cartons, serving primarily as secondary packaging materials (Cutter 2002; Marsh and Bugusu 2007).
Choosing the correct packaging material is an essential step in the processing of any food product. The key factors that must be considered vary with each food product and include the type of food being packaged, potential food-package interactions, intended consumer, length of shelf-life and distribution conditions (Marsh and Bugusu 2007). When processing a food product, the processing method, microbial target, shelf-life and type of package must be considered to result in a safe, high quality food product.

**Probiotics**

**Defining probiotics**

Spoilage and pathogenic microorganisms are not desired in food products as they deteriorate the product quality and can cause harm to the consumer. However, not all microorganisms are harmful to the consumer or food product and can be desired in foods. Microorganisms have been essential in food production, specifically fermentations, for thousands of years (Kopp-Hoolihan 2001). Consumption of these fermented foods has been linked to potential health benefits due to the microorganisms.

This concept first began to evolve around 1908 when Eli Metchnikoff, a Nobel- Prize winning scientist from Russia, suggested that the long life of Bulgarian peasants resulted from the consumption of fermented milk products (Kopp-Hoolihan 2001; Fioramonti and others 2003). The term probiotic, meaning “for life” in the Greek language, was first used in 1965 by Lilly and Stillwell to describe substances secreted by one microorganism which stimulates the growth of another microorganism (Lilly and Stillwell 1965; Schrezenmeir and de Vrese 2001; Fioramonti and others 2003; Quigley 2010). In 1974, Parker was the first to define probiotics in a way similar to the definition used today, stating that ‘probiotics are organisms and substances which contribute to the intestinal microbial balance’ (Parker 1974; Schrezenmeir and de Vrese
The probiotic definition continued to change, with Fuller (1989) suggesting that “probiotics are a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance (Fioramonti and others 2003). The descriptions of Parker (1974) and Fuller (1989) brought probiotics and probiotic research closest to the definition used today: probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO 2001).

**Requirements and stability of probiotics**

While several genera and species of beneficial bacteria exist, not all are considered probiotics. The species and strains most commonly used as probiotics include *Lactobacillus* and *Bifidobacteria*, along with *Streptococcus* and some nonpathogenic yeast (Fioramonti and others 2003; Isolauri and others 2004; Quigley 2010; Nagpal and others 2012). For a bacterial species and/or strain to be considered probiotic, there are several requirements that must be met. First and foremost, the probiotic must be non-pathogenic, non-toxic and safe to consume with no known harmful properties. Probiotics must also have resistance to gastric acidity and pancreatic secretions, adhere to epithelial cells, inhibit the adhesion of pathogenic bacteria, resist antibiotics, have tolerance to food additives, produce antimicrobial substances toward pathogens and have stability in the food or supplement preparation (Kopp-Hoolihan 2001; Fioramonti and others 2003). The species used most often as probiotics, *Lactobacillus* and *Bifidobacteria*, are known to resist gastric acid, bile salts and pancreatic enzymes, can adhere to colonic mucosa and readily colonize the intestinal tract (Fioramonti and others 2003). Within each species, the probiotic potential of different bacterial strains differs as every strain is unique and may have site-specific areas of adherence and specific immunological effects (Isolauri and others 2004).
The benefits of probiotics can only be attained by the consumer if the probiotics remain viable under a variety of storage conditions. In probiotic foods and supplements, the chosen strains must be able to withstand the processing conditions required and still possess their necessary properties afterwards while surviving in sufficient numbers (Del Piano and others 2006). The stability of probiotics is also influenced by the genus, species and strain biotype, as well as parameters such as water, temperature, pH, osmotic pressure, mechanical friction and oxygen (Del Piano and others 2006).

**The gastrointestinal tract and probiotics**

The target location within the host for probiotics to reach is the gastrointestinal tract, specifically the colon, to provide health benefits to the host. The colon itself is naturally populated with microbes, containing approximately 300-500 different bacterial species (Quigley 2010). However, the probiotics must survive the harsh conditions of the host digestive system and small intestine to reach the colon, which is a more favorable environment. The antimicrobial effects of gastric acid in the stomach and proximal small intestine make these areas unsuitable for most bacteria and contain relatively small numbers of bacteria in healthy individuals (Isolauri and others 2004; Quigley 2010). The upper bowel of the small intestine is sparsely populated with microorganisms, but as bacteria reach the ileum, the bacterial variety and concentration gradually increases, reaching levels of $10^{10}$-$10^{12}$ CFU/g or mL in the colon (Isolauri and others 2004; Del Piano and others 2006; Quigley 2010).

It is necessary for the probiotics to reach the colon as it is the main immunological organ in the human body (Dock and others 2004; Dock-Nascimento and others 2007). The intestines (gut) serve as the main barrier in the human body between external and internal environments to prevent potentially harmful compounds from entering the blood stream (Fioramonti and others...
2003). Among the various components of the intestines, the gut microflora is one of the main constituents of the intestinal defense barrier and is considered the first line of defense (Dock and others 2004; Dock-Nascimento and others 2007). Probiotics can contribute to the gut microflora and play a role in the immunological process by promoting gut barrier functions, giving maturational signals for the gut-associated lymphoid tissue and balancing the generation of pro- and anti-inflammatory cytokines. Together, these processes create healthy interactions between the host and probiotics which are necessary to properly regulate host inflammatory responses (Isolauri 2001).

**Potential benefits of probiotics**

Possible valuable health outcomes due to the use of probiotics range from broad influences within the host to playing a role in preventing and/or treating some diseases. General beneficial effects from probiotic consumption include: improvement of intestinal health by regulating/stabilizing the microflora and excluding pathogens, stimulation and development of the immune system, improvement of the nutritional quality of foods through increased bioavailability, stimulation of vitamin synthesis and enzyme production and enhancement of the host immune defenses through production of antimicrobial substances (Cano and others 2002; Nagpal and others 2012).

The mechanisms used by probiotics to produce health advantages are not completely known, but several mechanisms have been researched and suggested (Yan and Polk 2010). Some probable mechanisms include increased enzyme production, enhanced digestion and nutrient uptake and maintenance of the host microbial balance in the intestinal tract through production of bactericidal substances that compete with pathogens and toxins for adherence to the epithelium in the intestinal tract (Yan and Polk 2010). Other proposed mechanisms include
promoting intestinal epithelial cell survival, protective responses and barrier function, regulation of immune responses by enhancement of the innate immunity and prevention of pathogen-induced inflammation (Yan and Polk 2010).

Probiotics have also been associated with positive outcomes in some diseases, especially intestinal diseases. Clinical trial results for various probiotics have suggested that probiotics may have potential applications in the prevention and/or treatment of gastrointestinal disorders that include: inflammatory bowel disease, antibiotic associated diarrhea, irritable bowel syndrome, neonatal necrotizing enterocolitis, enteropathy in HIV infection, gluten intolerance, gastroenteritis, *Heliobacter pylori* infection and colon cancer (Yan and Polk 2010).

Of the gastrointestinal diseases diarrheal diseases, specifically infectious diarrhea, has been the focus of research. Infectious diarrhea is a worldwide problem in children and diarrheal diseases remain a leading cause of illness and death among infants and children in many developing countries (Saavedra 2000; Van Niel and others 2002). The preliminary results and clinical findings have shown that probiotics were an important therapy in the prevention and treatment of infectious diarrhea, such as traveler’s diarrhea, viral diarrhea, bacterial diarrhea and antibiotic associated diarrhea (Saavedra 2000; Isolauri 2001). Hilton and others (1997) found significantly reduced rates of diarrhea among 245 people traveling from the United States to developing countries when treated with *Lactobacillus* GG starting two days before travel (Saavedra 2000). Reviews of the use of probiotics as a prophylactic treatment for traveler’s diarrhea based on double-blind, placebo-controlled studies suggest that some strains of lactic acid bacteria may protect against traveler’s diarrhea (Isolauri 2001). A meta-analysis by Van Niel and others (2002) found that *Lactobacillus* is a safe, effective treatment for infectious
diarrhea in children as diarrhea duration was reduced by approximately two thirds of a day and diarrhea frequency was reduced on the second day by one to two stools.

Research has also shown that probiotics may play a role in the treatment of other diseases and conditions. Probiotics have exhibited some positive outcomes in the treatment of allergies, which is thought to be due to probiotics preventing antigen translocation in the blood stream and/or prevention of excessive immunologic responses to increased levels of antigen. Treatment of urogenital infections with probiotics has also had positive outcomes, with the probiotics adhering to the urinary and vaginal tract cells and competitively inhibiting the harmful bacteria. Research has also shown that probiotics are valuable in the control of blood lipids and heart disease by providing an antioxidative effect and by assimilating cholesterol into bacterial cells (Yan and Polk 2010; Nagpal and others 2012). While beneficial outcomes have been found with the use of probiotics in various diseases and conditions, research is still needed to determine the exact effects, strains and doses needed.

**Malnutrition and probiotics**

As described above, malnutrition is a condition that causes widespread health problems with short and long term consequences, including injury to the intestinal tract affecting nutrient absorption and immune response (Cano and others 2002; Guerrant and others 2008). Nutritional deficiencies are often connected with an impaired immune response, specifically cell-mediated immunity, phagocyte function, cytokine production, secretory antibody response and antibody affinity (Cano and others 2002). In protein energy malnutrition, a majority of the host defense mechanisms are breached, depending on the severity of the protein deficiency in relation to energy, which allows microbes to invade the host intestinal tract resulting in clinical infections that are often more severe and prolonged (Cano and others 2002). Protein malnutrition also
disturbs the normal ecology of microflora which can lead to an overgrowth of certain potentially harmful microorganisms (Dock-Nascimento and others 2007).

Several studies have looked at the effects of different probiotics on recovery of the intestinal tract in malnourished patients. Cano and others (2002) observed the effects of treatment with and without *Lactobacillus casei*, along with a standard renutrition diet, in malnourished mice and found positive biological effects in the immunosuppressed malnourished mice. In a separate study, *Streptococcus thermophilus* and *Lactobacillus helviticus* were used in combination in malnourished mice and the results showed ileum villi height in the intestine was shorter in malnourished mice compared to those treated with probiotics (Dock and others 2004). The study also found that other measurements of the intestines (crypt depth and wall width) were lower in malnourished mice compared to healthy mice (Dock and others 2004). Galdeano and others (2011) used fermented milk containing *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus casei* as one renutrition treatment in mice and found that mice given these treatments had higher counts of bifidobacteria and IgA+ cells, which are important in host immunity.

Results from studies using probiotics in malnourished mice suggest that there may be some benefit to providing probiotics to malnourished individuals along with the needed nutrients. Few studies have been conducted in malnourished children in developing countries to look at the benefit of probiotics in malnutrition recovery. One study conducted in Malawi recruited 1,024 children with severe acute malnutrition. The children were given a mixture of both probiotics, including *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Lactobacillus paracasei* subspecies *paracasei* and *Lactobacillus plantarum* and prebiotics or a placebo (Kerac and others 2009). The results of the study showed that the incidence of diarrhea did not differ
between the two groups, but those treated with probiotics had less severe bouts of diarrhea (Kerac and others 2009). The study also observed that deaths were similar between the two groups and the researchers concluded that the probiotic/prebiotic supplement did not improve the measured nutritional or clinical outcomes for severe acute malnutrition (Kerac and others 2009). However, further research is still needed. This study was conducted in children suffering from severe acute malnutrition, which represents the worst cases of malnutrition. It is still possible that probiotics may provide potential benefits to people with moderate acute malnutrition. The specific strains of probiotics used may also have an influence on the outcomes as each strain has unique properties (Isolauri and others 2004).

References


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CHAPTER 3
THE USE OF NUTRIENT OPTIMIZING/COST MINIMIZING SOFTWARE TO DEVELOP
READY-TO-USE THERAPEUTIC FOODS FOR MALNOURISHED PREGNANT WOMEN
IN MALI¹

¹ Bechman, A, R.D. Phillips and J. Chen. To be submitted to Plant Foods for Human Nutrition
Abstract

Malnutrition affects people of all ages in many countries in the developing world. One treatment for malnutrition is the intervention involving ready-to-use therapeutic foods (RUTFs). The present study developed RUTFs, using formulation computer software and largely local, plant-based ingredients for pregnant women in Mali, a country with the 2nd highest birth rate and infant mortality rate. Nutrient profiles of possible ingredients and their prices from 2004 to 2009 were entered into the software. Chosen ingredients of six formulations included peanuts, cowpeas and millet as well as rice or barley koji (sources of α-amylases as well as ingredients) were milled and hydrolyzed with the α-amylases in koji. The products were heated at 121 °C for 15 min. The contents of protein, fat, ash, fiber, carbohydrates, amino acid and energy of dehydrated products were determined and compared with software predicted values. Results showed that the actual and predicted values were comparable: the protein content was 1.45-2.04% higher, and ash content was 0.60-0.80% higher than the predicted values, while the fat content was 0.18-0.88% lower, the lysine content was 0.17-0.25% lower, and fiber content was 0.16% lower to 2.06% higher than the predicted values. The actual and predicted energy levels varied more significantly, from 11.10 to 19.70%. The amount of RUTF needed to meet the requirement of most limiting nutrients, lysine and energy, ranged from 2,620 to 3,002 g. The costs for producing the RUTFs were substantially lower than importing commercial RUTFs even with increased ingredient prices in Mali from 2004 to 2009.
Introduction

A healthy population is one of a country’s most valuable resources, as healthy citizens are able to contribute to both society and the economy. However, acute diseases and chronic conditions may threaten the health of individuals and large segments of the population, especially in developing countries. Malnutrition, which results from diets that do not provide sufficient energy and essential nutrients, is a chronic condition that impacts much of the developing world [1].

While malnutrition is commonly associated with children, people of all ages are susceptible, especially pregnant women. Research has shown that pregnant women suffering from malnutrition have greater maternal morbidity and mortality compared to healthy individuals [2]. Pregnancy malnutrition threatens the health of not only the mothers but also their unborn children [3]. Pregnant women affected by malnutrition are at a greater risk of morbidity, specifically infections and anemia, and mortality [2]. Untreated malnourished mothers during pregnancy are at a high risk of giving birth to an infant who is smaller, weaker, less resistant to disease and less intelligent compared to infants born to well-nourished mothers [4]. Infants born to malnourished women also tend to have a high mortality rate and impaired physical development [5]. These babies are very likely to become malnourished adults themselves, in an intergenerational cycle of malnutrition [1].

One successful treatment for malnutrition in children is the intervention involving ready-to-use therapeutic foods, known as RUTFs. RUTFs are energy- and macronutrient-dense foods fortified with vitamins and minerals [6]. Most previously available RUTF formulations are made of peanuts, powdered sugar, oil, powdered milk, vitamins and minerals [7]. An important advantage of RUTFs, other than their nutritional benefit, is that they require no preparation after
processing, allowing for home treatment of moderate cases of malnutrition rather than hospitalization [8].

However, the most current RUTFs contain powdered milk, which is not commonly available in most developing countries, making the RUTFs expensive to produce and difficult to access, especially for poor populations in the developing countries [9,10]. These products also contain a high percentage of peanut paste which has a thick consistency, making them difficult to swallow for some individuals [9]. Research has shown that thickened nutritious beverages are better alternatives for patients who have difficulty in eating and swallowing since the products hydrate the oral cavity and reduce the speed of liquid flow through the digestive tract, particularly in the pharyngeal region [11].

Utilization of local ingredients in a country or region in the RUTF could reduce the total cost of RUTFs. The staple ingredients in West Africa are comprised of cereals, legumes, oilseeds and starchy roots and tubers which must be combined in proper amounts to provide the nutrients pregnant women need [12]. Utilizing mathematical models, linear programming techniques have been used in the animal feed industry to produce favorable formulations while minimizing costs [10, 13]. Linear programming has been used in human nutrition since 1959 and can be used to assess the economic value of fortified food supplements and predict limiting nutrients in a developed formulation, making it a suitable tool for development of RUTFs [10, 14].

Most available RUTFs have been designed for treatment of malnutrition in children and may not be optimal for pregnant women who have different nutritional requirements. In general, the nutritional status of West African women is poor, with 13-20% of women of child-bearing age having a Body Mass Index indicative of chronic energy deficiency [15]. The objective of
this research was to develop low-cost, plant-based RUTFs targeting malnourished pregnant women in Mali, a West-African country with the 2nd highest birth rate and infant mortality rate in the world [16].

**Materials and methods**

*Ingredients and their nutrient profile and cost*

Corn (maize), sorghum, ground nuts (peanuts), millet, fonio, cassava, cowpeas, rice, barley, yams, sugar and sesame were chosen as potential ingredients of the RUTFs, and a vast majority of these ingredients were locally available and commonly consumed in Mali [17, 18, 19]. These ingredients were selected based on research conducted showing the foods commonly consumed in Mali and a list of the agricultural commodities with the highest production rates [20]. Representative nutrient profiles of all ingredients were obtained from the USDA Nutrient Database for Standard Reference [21] except for the profile of fonio, which was from a journal article [22]. The prices of all ingredients except sugar during the period of 2004 to 2009 were obtained from FAO PRICESTAT and are displayed in Table 3.1 [23]. A single price of sugar was obtained from a Malian newspaper article (Table 3.1) [24].

Along with identifying potential ingredients for RUTFs, an enzyme source was also needed in the processing of the RUTFs. Rice and barley fermented with *Aspergillus oryzae* (termed rice and barley koji) was made and used in the production of the RUTFs. Koji usually contains several enzymes including α- and β-amylases, glucoamylases, α-glucosidase, and acid and neutral proteases [25]. Amylase activities of the koji used in this study were determined by the 3, 5-dinitrosalicylic acid method as described by Miller [26] with modifications. Each gram of rice or barley koji used in the present study had 59.78 or 117.46 U (expressed as mg maltose released/g koji solids/min) of α-amylases [27]. Protease contents of the rice and barley koji were
found to be very low [27]. The amylases were used to hydrolyze the starch in the products, reducing the viscosity/thickness of the RUTFs, making it easier to swallow and making nutrients easier to absorb by malnourished individuals. However, the nutrient composition of the fermented products was not available, and therefore the nutrient profile of unfermented grains was used.

Computer software and product formulation

Formulation computer software was used to develop the RUTFs (CFC4-S2®; Creative Formulation Concepts, LLC, Annapolis, MD, USA). The software uses a set of linear equations to develop a formulation optimized for the nutrient profile desired while minimizing the cost. All potential ingredients along with their nutrient profiles and prices were entered into the software. The nutrient requirements of pregnant women set by the Food and Agriculture Organization and the Institute of Medicine/US National Academy of Sciences (Dietary Reference Intakes) were used as references to develop the RUTFs (Table 3.2) [28, 29].

The formulation software allows restrictions to be placed on both the ingredients and the level of nutrients used in the RUTFs. Restrictions placed on nutrients helps ensure that the RUTFs provide necessary nutrition to the target population in each serving. Restrictions on the ingredients can be used to improve the palatability of the products by limiting excess amounts of certain ingredients when necessary. In the present research, restrictions were placed on both nutrients and ingredients in order to develop nutritionally desirable RUTFs. Table 3.2 shows the nutrient restrictions applied in this optimization. A maximum restriction of 20% was set for the total fat content based on the Acceptable Macronutrient Distribution Range [30]. A minimum of 452 kcal/100 g was specified to ensure a high energy content and minimum levels of essential amino acids based on the reference pattern for 1-3 y old children were specified. Daily amino
acid requirements by pregnant women were unavailable [28, 29], therefore the amino acid scoring pattern for 1-3 y old children was used. Like toddlers, pregnant women have high nutrient requirements due to increased physiologic demands, and therefore, formulation of RUTFs with this approach will provide a sufficient level of amino acids for the target population [29]. Sugar was restricted to a maximum level of 14% based on results of preliminary formulations to balance the nutrient values of the RUTFs. No restrictions were placed on micronutrients because a vitamin/mineral premix will later be added to these base formulations to make complete RUTFs. Restrictions were also placed on the amount of rice (7, 14 or 21%) or barley (5, 10 or 15%) koji used in the products. The levels of restriction were based on the amount of α-amylases and their activities needed to breakdown at least 50% of the starch in the RUTFs in the incubation time utilized. The formulation software also allows for the ingredient groups, such as cereals and legumes, to be specified in order to develop a formulation with a complete, balanced protein content.

Once all necessary information was entered into the software, formulations were developed using the ingredient price data during the period of 2004 to 2009 in order to determine the influence of commodity price on the final ingredient cost of the RUTFs.

Processing of RUTFs

Based on the above restrictions described (Table 3.2) and the ingredient prices (Table 3.1), 6 RUTF products were formulated and processed. The selected ingredients were obtained from sources in Georgia: blanched, roasted peanuts from American Blanching Company (Fitzgerald, GA, USA), cowpeas and millet flour from Dekalb Farmers Market (Decatur, GA, USA), rice and barley from Sevananda Natural Foods Market (Atlanta, GA, USA) and sugar and salt from local retail stores (Griffin, GA, USA). Decorticated cowpeas and millet flour were
boiled in tap water (1:10), and then mixed with roasted peanuts. Rice (7, 14 or 21% of the formulation) and barley (5, 10 or 15% of the formulation) koji produced according to the methods of Bechman et al. [27] were added. According to these formulations, a 100 g of RUTFs containing the rice koji (dry weight) had 418.46, 836.92 or 1255.38 U of α-amylases while the same amount of products with barley koji (dry weight) had 587.30, 1175.00 and 1761.69 U of the enzymes [27]. After the addition of koji, additional water (71.67-244.6 mL) was added, and diluted mixtures were passed through a colloid mill (Morehouse Industries, Los Angeles, CA, USA) twice. The amount of water added was determined by subtracting the water used for boiling the cowpeas and millet flour from the total batch size of a product intended to prepare. The products were then incubated for 4.5 h at 55 °C in a reciprocal shaking water bath (ThermoScientific, Marietta, OH, USA) for starch hydrolysis. Digested products were subsequently boiled on a stove (Amana, Benton Harbor, MI, USA) for 10 min to inactivate the α-amylases, followed by the addition of sugar and salt. The products were then filtered through a 2 mm sieve (Fisher Scientific, Pittsburgh, PA, USA), dispensed into glass bottles (160 mL; Fisher Scientific), and heated at 121°C for 15 min (Steris, Mentor, OH, USA). Heated products were cooled, and then stored at 4 °C for nutrient analysis.

Nutrient Analysis

After processing, sub-samples of the RUTFs were freeze-dried (The Virtis Company Inc, Gardiner, NY, USA) in plastic containers (5.5x5.5x2 in; Rubbermaid, Atlanta, GA, USA) covered with aluminum foil (Fisher Scientific; with punched holes). The products were frozen to -20 °C and then dried for 18 h at 20 ± 2 °C. Protein analysis was performed using the combustion method [31]. Total amino acid profiles of the RUTFs were analyzed using acid hydrolysis, followed by derivatization and separation using high performance liquid
chromatography [32]. The total fat content of the products was determined using the Goldfisch extraction method of AOAC 948.22 [31]. Ash contents were examined using the incineration method of AOCS (Ba 5-49) [33]. Moisture contents of all samples were analyzed using the vacuum oven drying method of AOAC 925.10 [31] while total dietary fiber was evaluated using the enzymatic-gravimetric method of AOAC 985.29 [31]. Total carbohydrate was calculated by difference, and the energy (kcal) was calculated as the sum of the total amount of protein, fat and carbohydrate (g) in an individual RUTF multiplied by the amount of energy provided by a unit dry weight of each nutritional component (4 kcal/g of protein and carbohydrates; 9 kcal/g of fat) [34]. Based on obtained nutrient values and the assumption that the RUTFs are the only source of nutrients, the amount of RUTFs that has to be consumed in order to meet the daily nutrient requirements of a pregnant woman was determined. The daily requirement for each nutritional component was divided by the actual nutrient content in unit dry weight of the RUTFs.

Statistical analysis

Data obtained was analyzed using Statistical Analysis Software (version 9.1; Cary, NC, USA). A two-way analysis of variance (ANOVA) along with Fisher’s least significant difference test was used to determine the significance of difference in product protein, fat, ash, fiber, carbohydrate, amino acid, and energy values based on a confidence level of 95%.

Results and Discussion

Formulation and analysis of computer-generated RUTFs

The amounts of peanut and cowpea varied in the 6 selected RUTF formulations (Table 3.3). However, the differences in the amounts of the two components were small, about 1.32 and 1.75%, respectively. The greatest variation among the 6 formulations was the content of millet
flour, varying about 16.80%. In general, the amount of millet flour decreased as the percentage of koji in the RUTFs increased (Table 3.3).

Table 3.4A shows the actual macronutrient and energy contents of the 6 RUTF products. It is observed that software prediction for the nutritional components of the RUTFs was reasonably accurate. The actual protein contents of the products were 1.45-2.04% higher, while the actual fat contents were 0.18 to 0.88% lower than the predicted values (Table 3.4A). Differences between predicted and actual ash contents were 0.60-0.80% (Table 3.4A). The fiber content of the RUTFs containing rice koji varied from -0.16 to 0.49% from predicted values, while that of the barley koji-containing RUTFs was 0.92-2.06% lower than the predicted values. Greater differences (11.10-19.70%) were noticed between predicted and actual energy contents of the RUTFs (Table 3.4A).

Some of the actual nutrient components of the RUTFs including protein, fat, ash and fiber were similar to the predicted values, suggesting that the formulation software can be used to develop nutrient dense/cost optimized RUTFs for pregnant women. The observed variations were probably due to the differences between reference and actual nutrient values of the ingredients used in the study. The USDA nutrient data on the RUTF ingredients was based on raw, whole seeds [21, 22]. Different forms of ingredients were, however, used in the present study: the cowpeas were decorticated, peanuts were roasted, rice and barley were fermented, and millet flour instead of millet seed was used. Furthermore, the nutrition profiles of the RUTF products reported in the present study were determined after the final step of processing followed by product dehydration. All of these processing steps might have an impact on the nutritional composition of the product (Table 3.4A).
Variations between computer software predicted and actual nutritional compositions of food products have been observed and reported in previous studies. El-Hebashy et al. [35] observed that the actual protein content of weaning foods developed using formulation software were either 0.4% lower or 2.1% higher, the fat content was 1.1-1.7% higher, and the ash content was 0.1-0.5% higher than predicted values. A RUTF developed by Dibari et al. [10] for malnourished adults with HIV had an energy content that was 15.76% higher than predicted values. Furthermore, protein content of the product was 2.3% higher, and the fat content was 1.0% lower than predicted values.

The commercially available RUTF often used in the treatment of malnutrition, specifically in children, is PlumpyNut®. This RUTF is made of peanut butter, sugar, oil and non-fat dry milk (NFDM), along with a vitamin/mineral premix [36]. It has been reported that each 100 g of PlumpyNut® contains 545.00 kcal, 13.60 g protein and 35.70 g fat [36]. Comparing the same amount of RUTFs (dry weight) developed in the present work to the commercially available PlumpyNut®, the present RUTFs contain ~1.30-1.50 fold more protein, but ~1.80 fold less fat and 1.10-1.20 fold less energy [36]. The difference in the fat content and subsequently the energy content between the current RUTFs and PlumpyNut® is that the latter contains approximately 15% of vegetable oil [9]. Furthermore, the fat content of the RUTFs developed in the present study was restricted in order to balance the overall nutrition profile of the formulations to meet the needs of malnourished pregnant women. The fat content in the formulations could be raised, by the addition of oil for example, which would result in a higher fat and energy content without compromising the availability of other nutrient components.

Comparing the present RUTFs to PlumpyNut® is beneficial as it is the commercially available RUTF and its composition is the one commonly used in the treatment on malnutrition.
However, since it is designed to treat malnutrition in children, it is necessary to evaluate the RUTFs developed in this work to products designed for similar target groups in the literature. Bahwere et al. [37] formulated a RUTF for malnourished HIV-positive adults in Malawi using local ingredients and excluding NFDM that consisted of sesame seeds, chickpeas, corn, vegetable oil, sugar and vitamins and minerals (CS-RUTF) [37]. Malnourished, HIV-positive females, a target group of Bahwere et al. [37], require 2,600-2,820 kcal and 48 g of protein to maintain weight, which is similar to the 2,615.20 kcal needed by pregnant women, the target group in the present work, during the third trimester of pregnancy (Table 3.2) [28]. The nutrient composition of the CS-RUTF contained 536.2 kcal/100 g energy and 12.3 g/100 g protein [37]. When comparing the present RUTFs to the CS-RUTF, the RUTFs in this work had a higher protein content and a lower energy content, as was also seen when evaluating these RUTFs with PlumpyNut®. The lower content in the RUTFs here is again attributed to the use of vegetable oil in the CS-RUTF as well as the nutrient restrictions placed in the formulation software in this work to develop balanced RUTFs.

The amino acid profiles of the 6 RUTF products are shown in Table 3.4B. It is observed that the levels of several essential amino acids including lysine, histidine, threonine and cysteine fell slightly below software predicted values in all 6 products. Formulation C had 6 amino acids, while products A, B and D each had 4 amino acids whose values were lower than software predictions (Table 3.4B). However, the differences between predicted and actual amino acid contents were small, ranging from 0.03 to 0.25% of the formulations (Table 3.4B). Two products (C and F) also had a lower (0.01%) than predicted tryptophan content, and product C also had a lower (0.01%) than predicted valine content. The greatest difference was observed with the predicted and actual lysine content of the 6 RUTFs, ranging from 0.17 to 0.25%.
The formulation software created nutritious products that satisfied the protein, and 5 to 7 essential amino acids, required by pregnant women (Table 3.4B). This suggests that cereals and legumes can be used for the development of nutrient dense foods without the use of NFDM. However, the exclusion of NFDM from the current RUTFs did have an impact on the protein and amino acid profiles, specifically the lysine content of the RUTFs because 100 g of NFDM contains 36.16 g of protein and 2.68 g of lysine [21]. The elimination of NFDM may also influence the protein quality of the RUTFs. Plant proteins have been shown to have a lower quality compared to animal based proteins [38]. A high quality protein mixture can be achieved solely using plant ingredients if complementary proteins are combined [38]. In the present research legumes, peanuts and cowpeas, and cereals, millet, rice and barley, were mixed to provide a balanced, complete protein.

Despite the use of complementary proteins, the current protein and amino acid profiles of the current RUTFs could be improved through the inclusion of other higher quality plant ingredients such as soybeans, 100 g of which contain 36.49 g of protein and 2.71 g of lysine [21]. Soybean production is currently limited in Mali although Nigeria, another country in West Africa, led African soybean production in 2007 [17, 39]. The potential of using soybeans to improve the protein quality and subsequently amino acid profiles of the RUTFs should be explored.

In addition to the choice of ingredients, processing conditions could also have an impact on the contents of essential amino acids. Chemical reactions, specifically Maillard browning during processing, may occur which can reduce the overall amino acid contents of the products. A previous study showed that when glucose/fructose-casein solutions (pH 7) were heated at 120 °C for 40 min, the number of lysine residues decreased from ~16 to ~8 mmol/l [40]. In the
present study, the RUTFs were heated at 121 °C for 15 min, which could have induced Maillard browning and contributed to the variations between actual and software predicted amino acid values. However, the thermal process used in the present study is essential for the production of safe and microbiologically stable products. To reduce the severity of the heat treatment other antimicrobial hurdles will have to be considered.

**Calculated amount of RUTFs needed to meet daily nutrient requirements**

In order to meet the daily requirement of carbohydrate, fat and protein shown in Table 3.2, approximately 578-610 g, 1,390-1,444 g or 1,704-1,784 g (wet weight) of RUTFs will have to be consumed. This calculation was based on the assumption that the RUTFs were the only food source in the diet of a malnourished pregnant woman. To meet the requirements for energy or lysine (Table 3.2) approximately 2,656-2,734 g or 2,620-3,002 g of RUTFs will have to be consumed (Figure 3.1). Since lysine is the most limiting nutrient in all 6 RUTFs, consuming an adequate amount of products to meet the requirement of lysine will also supply sufficient amounts of macronutrients, energy and other limiting amino acids such as histidine, threonine, cysteine, tryptophan and valine.

The RUTFs developed in this research can be used to provide various amounts of each nutrient, depending on the severity of malnourishment of each pregnant woman and the level of nutrients acquired from other food sources. As mentioned previously, in order to meet the energy requirement of 2,615.20 kcal/day (Table 3.2), a pregnant woman would need to consume 2,656-2,734 g of products developed in the present study (Figure 3.1). RUTFs developed by Nabuuma et al. [41] targeted young children and to provide the 1,400 kcal/day of energy needed by a 15.4 lb child (7 kg), approximately 1,257-1,386 g would be required per day. However, the amount of product that can be consumed per day by a patient is dependent on the severity of
malnutrition and a person’s willingness to consume the product. In a 3 month clinical trial in Malawi, HIV positive patients with a BMI indicative of malnutrition were given 500 g/day of the CS-RUTF described above [37]. However, an average daily intake of 300 g was recorded [37]. Even with lower than expected daily intake, weight gain was observed in 73.3% of the participants [37]. This suggests that weight gain can be accomplished even if a portion of the product is consumed. The amount of necessary RUTF consumption can be adjusted based on a patient’s needs, allowing treatment for malnutrition to be easily customized with the same formula.

Price trend of RUTFs

Table 3.5 shows predicted costs required to process a unit weight of the RUTF products based solely on the average yearly ingredient price during the period of 2004 to 2009. It is observed that the costs for producing any of the 6 RUTFs increased from $0.33/kg dry product in 2004 to $0.50/kg dry product in 2009 with a total change of $0.17/kg dry product due to the rise in ingredient prices (Table 3.5).

However, the price for producing RUTFs is influenced by the cost of both processing and ingredients. UNICEF reports showed that 68% of the overall cost of PlumpyNut® comes from ingredient purchases [42]. The price of imported PlumpyNut® in Kenya in 2008 was approximately $5.00/kg [43]. This suggests that $3.40/kg of the final price of the imported PlumpyNut® was spent on ingredients [42, 43]. Therefore, regardless of the processing costs, reducing the expenses on ingredients will significantly reduce the final cost of a RUTF [42, 43]. Dibari et al. [9] developed a RUTF for the region of East Africa using maize, soy, sorghum, palm olein oil and sugar that was ~$0.70/kg. The ingredient costs for the present RUTFs ranged from $0.33 to $0.50/kg dry product which, along with previous research, suggests that even with
added processing and packaging costs the total production cost for the current RUTFs are likely to be substantially lower than that of the PlumpyNut® [10].

4. Conclusions

The formulation computer software can be utilized to develop nutrient dense, cost effective RUTFs based largely on local, plant-based ingredients for malnourished pregnant women in Mali. The actual protein, fat, ash, fiber and amino acid contents of the developed RUTFs were comparable with computer software predicted values. Energy contents of the RUTFs were 11.10 to 19.70% away from predicted values. If the RUTFs are the only source of nutrients for a malnourished pregnant woman, 2,620-3,002 g of the products developed in the present study are needed in order to meet the daily requirement for the most limiting nutrients, energy or lysine. However, the amount of RUTF needed to meet daily nutrient requirements can be adjusted, depending on the other sources of nutrients in the diet. The fat and energy contents of the products can be increased through the use of oils and/or changing the level of restriction for fat content during product formulation. Once fortified with multi vitamins and minerals the products will satisfy the overall nutrient requirement of pregnant women in their third trimester.

The costs for producing the present RUTFs varied each year during the period of 2004 to 2009 due to increases in ingredient prices but were substantially lower than imported commercial RUTFs. The formulation computer software can be utilized to develop nutrient dense, cost effective RUTFs based largely on local, plant-based ingredients for malnourished pregnant women in Mali. The actual protein, fat, ash, fiber and amino acid contents of the developed RUTFs were comparable with computer software predicted values. Energy contents of the RUTFs were 11.10 to 19.70% higher than predicted values, providing a higher energy density per 100 g dry basis of RUTF than originally expected. If the RUTFs are the only source of
nutrients for a malnourished pregnant woman, 2,656-2,734 g of the products developed in the present study are needed in order to meet the daily requirement for the most limiting nutrient, energy. However, the amount of RUTF needed to meet daily nutrient requirements can be adjusted, depending on the other sources of nutrients in the diet. The fat and energy contents of the products can be increased through the use of oils and/or changing the level of restriction for fat content during product formulation. Once fortified with multi vitamins and minerals the products will satisfy the overall nutrient requirement of pregnant women in their third trimester. The costs for producing the present RUTFs varied each year during the period of 2004 to 2009 due to increases in ingredient prices but were substantially lower than imported commercial RUTFs.

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References:


Table 3.1 Prices for potential RUTF ingredients ($USD/kg) over a 6 year period in Mali\textsuperscript{a}

<table>
<thead>
<tr>
<th>Year</th>
<th>Corn</th>
<th>Sorghum</th>
<th>Millet</th>
<th>Barley\textsuperscript{b}</th>
<th>Rice</th>
<th>Peanuts</th>
<th>Cowpeas</th>
<th>Yam</th>
<th>Cassava</th>
<th>Fonio</th>
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</tr>
<tr>
<td>2007</td>
<td>$0.15</td>
<td>$0.18</td>
<td>$0.18</td>
<td>$0.22</td>
<td>$0.25</td>
<td>$0.40</td>
<td>$0.30</td>
<td>$0.31</td>
<td>$0.11</td>
<td>$0.63</td>
<td>$1.03</td>
</tr>
<tr>
<td>2008</td>
<td>$0.17</td>
<td>$0.19</td>
<td>$0.19</td>
<td>$0.23</td>
<td>$0.26</td>
<td>$0.47</td>
<td>$0.36</td>
<td>$0.28</td>
<td>$0.09</td>
<td>$0.83</td>
<td>$1.03</td>
</tr>
<tr>
<td>2009</td>
<td>$0.18</td>
<td>$0.24</td>
<td>$0.29</td>
<td>$0.34</td>
<td>$0.32</td>
<td>$0.51</td>
<td>$0.35</td>
<td>$0.23</td>
<td>$0.06</td>
<td>$0.83</td>
<td>$1.03</td>
</tr>
</tbody>
</table>

\textsuperscript{a}[23]

\textsuperscript{b}Price data on barley from Mali was not available, so data from Algeria, a African country bordering Mali, was used.

\textsuperscript{c}One price for sugar was found for Mali based on a 2009 newspaper article and was used for all 6 years [24].
Table 3.2. Daily nutrient requirements and formulation restrictions for macronutrients and amino acids for pregnant women in their 3rd trimester.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcal/day&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2615.20</td>
</tr>
<tr>
<td>Protein (g/day)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.00</td>
</tr>
<tr>
<td>Carbohydrate (g/day)</td>
<td>65.00</td>
</tr>
<tr>
<td>Fat (g/day)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.12</td>
</tr>
<tr>
<td>Amino Acids (g/day)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>3.26</td>
</tr>
<tr>
<td>Leu</td>
<td>3.52</td>
</tr>
<tr>
<td>Val</td>
<td>2.05</td>
</tr>
<tr>
<td>His</td>
<td>1.15</td>
</tr>
<tr>
<td>Trp</td>
<td>0.45</td>
</tr>
<tr>
<td>Thr</td>
<td>1.73</td>
</tr>
<tr>
<td>Ile</td>
<td>1.60</td>
</tr>
<tr>
<td>Met + Cys</td>
<td>1.60</td>
</tr>
<tr>
<td>Phe + Tyr</td>
<td>3.01</td>
</tr>
</tbody>
</table>

<sup>a</sup>[28]
<sup>b</sup>[29]
<sup>c</sup>[29]
Table 3.3. RUTF formulations generated using Creative Concepts Formulation software.

<table>
<thead>
<tr>
<th>Ingredients (%)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanuts</td>
<td>38.41</td>
<td>38.95</td>
<td>39.49</td>
<td>38.17</td>
<td>38.46</td>
<td>38.75</td>
</tr>
<tr>
<td>Cowpeas</td>
<td>22.23</td>
<td>21.81</td>
<td>21.40</td>
<td>21.92</td>
<td>21.20</td>
<td>20.48</td>
</tr>
<tr>
<td>Millet</td>
<td>18.36</td>
<td>11.24</td>
<td>4.11</td>
<td>20.91</td>
<td>16.34</td>
<td>11.77</td>
</tr>
<tr>
<td>Sugar</td>
<td>14.00</td>
<td>14.00</td>
<td>14.00</td>
<td>14.00</td>
<td>14.00</td>
<td>14.00</td>
</tr>
<tr>
<td>Rice</td>
<td>7.00</td>
<td>14.00</td>
<td>21.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Barley</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.00</td>
<td>10.00</td>
<td>15.00</td>
</tr>
</tbody>
</table>

Formulations with Rice Koji          Formulations with Barley Koji
Table 3.4. Software predicted and actual macronutrients (A) and amino acids (B) of the RUTFs (on a dry weight basis).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Formulations with Rice Koji</th>
<th>Formulations with Barley Koji</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predicted Values</td>
<td>Actual Values</td>
</tr>
<tr>
<td>Protein (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>17.62</td>
<td>19.48</td>
</tr>
<tr>
<td>B</td>
<td>17.34</td>
<td>19.29</td>
</tr>
<tr>
<td>C</td>
<td>17.06</td>
<td>18.51</td>
</tr>
<tr>
<td>Fat (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>20.01</td>
<td>19.33</td>
</tr>
<tr>
<td>B</td>
<td>20.01</td>
<td>19.13</td>
</tr>
<tr>
<td>C</td>
<td>20.01</td>
<td>19.15</td>
</tr>
<tr>
<td>Ash (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.25</td>
<td>2.95</td>
</tr>
<tr>
<td>B</td>
<td>2.06</td>
<td>2.81</td>
</tr>
<tr>
<td>C</td>
<td>1.87</td>
<td>2.76</td>
</tr>
<tr>
<td>Fiber (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>7.18</td>
<td>6.90</td>
</tr>
<tr>
<td>B</td>
<td>6.58</td>
<td>6.42</td>
</tr>
<tr>
<td>C</td>
<td>5.97</td>
<td>6.46</td>
</tr>
<tr>
<td>Carb (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>52.47</td>
<td>51.34</td>
</tr>
<tr>
<td>B</td>
<td>52.67</td>
<td>52.36</td>
</tr>
<tr>
<td>C</td>
<td>52.87</td>
<td>53.13</td>
</tr>
<tr>
<td>Energy (kcal/100g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>441.26</td>
<td>456.12</td>
</tr>
<tr>
<td>B</td>
<td>441.20</td>
<td>458.11</td>
</tr>
<tr>
<td>C</td>
<td>441.13</td>
<td>460.83</td>
</tr>
<tr>
<td>Amino Acid (%)</td>
<td>Predicted Values</td>
<td>Actual Values</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Lys</td>
<td>0.77</td>
<td>0.59</td>
</tr>
<tr>
<td>Leu</td>
<td>1.34</td>
<td>1.42</td>
</tr>
<tr>
<td>Val</td>
<td>0.80</td>
<td>0.82</td>
</tr>
<tr>
<td>His</td>
<td>0.47</td>
<td>0.41</td>
</tr>
<tr>
<td>Thr</td>
<td>0.19</td>
<td>0.20</td>
</tr>
<tr>
<td>Ile</td>
<td>0.67</td>
<td>0.58</td>
</tr>
<tr>
<td>Met</td>
<td>0.25</td>
<td>0.27</td>
</tr>
<tr>
<td>Cys</td>
<td>0.23</td>
<td>0.21</td>
</tr>
<tr>
<td>Met+Cys</td>
<td>0.48</td>
<td>0.47</td>
</tr>
<tr>
<td>Phe</td>
<td>0.95</td>
<td>1.00</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.65</td>
<td>0.76</td>
</tr>
<tr>
<td>Phe+Tyr</td>
<td>1.60</td>
<td>1.76</td>
</tr>
</tbody>
</table>
Table 3.5. Changes in ingredient costs ($US/kg of dry product) of RUTF production due to increases in commodity prices in Mali from 2004 to 2009.

<table>
<thead>
<tr>
<th>Year</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>0.33</td>
<td>0.34</td>
<td>0.34</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33±0.006</td>
</tr>
<tr>
<td>2005</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>0.35</td>
<td>0.35</td>
<td>0.36±0.005</td>
</tr>
<tr>
<td>2006</td>
<td>0.37</td>
<td>0.38</td>
<td>0.38</td>
<td>0.37</td>
<td>0.37</td>
<td>0.37</td>
<td>0.37±0.006</td>
</tr>
<tr>
<td>2007</td>
<td>0.41</td>
<td>0.42</td>
<td>0.43</td>
<td>0.41</td>
<td>0.41</td>
<td>0.41</td>
<td>0.42±0.006</td>
</tr>
<tr>
<td>2008</td>
<td>0.46</td>
<td>0.46</td>
<td>0.47</td>
<td>0.45</td>
<td>0.46</td>
<td>0.46</td>
<td>0.46±0.006</td>
</tr>
<tr>
<td>2009</td>
<td>0.49</td>
<td>0.50</td>
<td>0.50</td>
<td>0.49</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50±0.003</td>
</tr>
</tbody>
</table>
Figure 3.1. The amount of each RUTF (A-F, shown from left to right) that must be consumed to meet the energy, protein, lysine (most limiting amino acid), fat and carbohydrate requirements, based on the assumption that the RUTFs are the only source of nutrient in a pregnant women’s diet. The reference nutrient requirements for pregnant women in their 3rd trimester are: energy-2,615.20 kcal; protein-64.00 g; lysine-3.62 g; fat-58.12 g; and carbohydrates-65.00 g (Table 3.2).
Fig 3.1. Bechman et al.
CHAPTER 4
PROCESSING AND CHARACTERIZATION OF READY-TO-USE THERAPEUTIC FOODS
FOR MALNOURISHED PREGNANT WOMEN IN MALI

2 Bechman, A, R.D. Phillips and J. Chen. To be submitted to LWT-Food Science and Technology
Abstract

Ready-to-use therapeutic foods are energy, nutrient dense foods used to treat malnutrition. Using local plant-based ingredients and local production of the RUTFs reduces the cost, making the products affordable. The focus of this research was to process and characterize plant-based RUTFs for pregnant women in Mali. Six RUTF formulations were processed by boiling, milling, enzymatic hydrolysis and heating to 121 °C for 15 min. Viscosity, color, pH and particle size were analyzed to characterize the physical properties of the RUTFs. Enzymatic hydrolysis was achieved through the use of koji and bromelain in the following amounts: rice koji, 418, 837 or 1255 U/100 g RUTF dry basis; barley koji, 587, 1175 or 1762 U/100 g RUTF dry basis; and bromelain in four concentrations of 0, 0.01, 0.1 or 1%. Surface response models were developed to determine the optimum levels of koji and bromelain for the RUTFs: 21% rice koji with 1% bromelain and 6% barley koji with 0.72% bromelain. Viscosity was found to increase with the addition of bromelain but maintained a drinkable consistency. The vitamin/mineral premix used to provide a nutritionally complete RUTF was stable at storage temperatures of 4, 25 and 37 °C for 12 mo.
1. Introduction

Malnutrition is a condition affecting all age groups in most of the developing world that results from a poor diet lacking the energy and essential nutrients needed by the body (Briend & Nestel, 2005). A current treatment option for malnutrition is ready-to-use therapeutic foods (RUTFs), which are energy and nutrient-dense foods fortified with vitamins and minerals (Collins, Dent, Binns, Bahwere, Sadler, & Hallam, 2006). Most RUTFs used are a paste consisting of ground peanuts, powdered milk, vegetable oil, sugar and a vitamin/mineral premix (Manary, 2006). A main advantage of RUTFs is that they do not require additional preparation, allowing for home treatment rather than hospitalization for moderate cases of malnutrition (Linneman, Matilsky, Ndekha, Manary, Maleta, & Manary, 2007). The formulation of the RUTFs often used allows the RUTFs to be produced in developing countries using basic processing technologies (Manary, 2006). The current formulations are shelf stable, with a shelf life of three to four months in tropical climates since the RUTFs have a low water activity, limiting bacterial growth (Manary, 2006).

The current RUTFs provide several advantages in the treatment of malnutrition, but disadvantages do exist. RUTFs are effective in treating malnutrition, but were designed for treating children less than five years of age. However, all age groups are susceptible to malnutrition, especially nutritionally vulnerable groups including infants, children and women, specifically pregnant women (Lartey, 2008).

Previous work by Bechman, Phillips, & Chen (Chapter 3) focused on formulating semi-liquid RUTFs for pregnant women in Mali utilizing local, plant-based ingredients. Once formulated, six RUTFs were selected for processing, with the emphasis being processing technologies that can be found in Mali. Processing methods used in the preparation of common
Malian dishes include wet techniques such as steaming, boiling and frying, and the less common dry methods, including baking, grilling and roasting (MacLean & Insoll, 1999). Grinding is utilized to prepare cereal grains and vegetables before they are cooked and consumed (MacLean & Insoll, 1999; Toure, Coulibaly, Arby, Maiga, & Cairncross, 2011).

As mentioned previously, most RUTFs have a paste consistency, resulting in thick RUTFs that can be difficult to swallow for some weak, malnourished individuals. Patients with difficulty in swallowing solid foods are treated with beverages thickened with cereals, starches or gelatin (Germain, Dufresne, & Ramaswamy, 2006). A RUTF made only from cereals, legumes and oilseeds however, may form a stiff paste that is not drinkable. The viscosity of these thick pastes can be reduced through enzyme hydrolysis resulting in a thickened, drinkable RUTF (Nout & Ngoddy, 1997; Rolle, 1998).

The objective of this research was to process and characterize nutritionally complete semi-liquid RUTF formulations using simple technologies.

2. Materials and Methods

2.1 Formulation

Creative Formulation Concepts (CFC4-S2® Creative Formulation Concepts, LLC, Annapolis, MD, USA) computer software was utilized to develop RUTF formulations. Twelve local ingredients from Mali, corn, sorghum, ground nuts (peanuts), millet, fonio, cassava, cowpeas, rice, barley, yams, sugar and sesame, Mali were selected and the nutrient profile of each was entered into the software, along with the nutrient requirements of pregnant women (Bechman et al., Chapter 3). Six base RUTF formulations consisting of peanuts, cowpeas, millet and rice or barley (Table 4.1) were selected for processing and product characterization.
2.2 Processing of RUTFs

Utilizing simple technologies that could be found in Mali, a processing scheme was developed to produce the six RUTF formulations (Figure 4.1). The selected ingredients for the six formulations (Table 4.1) were obtained from sources in Georgia: blanched, roasted peanuts from American Blanching Company (Fitzgerald, GA, USA), cowpeas and millet flour from Dekalb Farmers Market (Decatur, GA, USA), rice and barley from Sevananda Natural Foods Market (Atlanta, GA, USA) and sugar from local retail stores (Griffin, GA, USA). Decorticated cowpeas and millet flour were boiled with tap water (1:10) separately, and then peanuts were added into the mixture. Rice (7, 14 or 21% of the formulation) or barley (5, 10 or 15% of the formulation) was added in the form of koji, serving both as an ingredient and a source of α-amylase (Bechman, Phillips, and Chen, 2012). The α-amylase concentration was 418, 837 or 1255 U in 100 g of RUTF dry basis for rice koji, with units expressed as mg maltose released/g koji solids/min (Bechman et al., 2012). The barley koji had an enzyme concentration of 587, 1175 or 1762 U/100 g RUTF dry basis (Bechman et al., 2012). After the addition of koji, water (72-245 mL) was added and diluted mixtures were passed two times through a colloid mill (Morehouse Industries, Los Angeles, CA, USA). The quantity of additional water added was determined by subtracting the water used for boiling the cowpeas and millet flour from the total batch size of a product intended to prepare. The milled product was divided into four 8 quart stainless steel cooking pots (Crate and Barrel, Northbrook, IL, USA), covered with glass lids and held at 55 °C for 4 h in a reciprocal shaking water bath at 50 rpm (ThermoScientific, Marietta, OH, USA) to allow the α-amylases to hydrolyze the starch. After 4 h, each RUTF mixture received one of the following levels of the protease, bromelain (Kalyx, Camden, NY, USA): 0, 0.06, 0.6 or 6 Gelatin Digesting Units/g (Kalyx, 2012). Following the addition of bromelain, the
RUTFs were incubated for 30 min in the water bath at 55 °C with a shaking speed of 50 rpm. The product was then boiled for 10 min on a stove (Amana, Benton Harbor, MI, USA) to inactivate enzymes, followed by the addition of sugar and salt (Wal-mart, Griffin, GA, USA). The product was filtered through a 2 mm sieve (Fisher Scientific, Pittsburgh, PA, USA), filled into 160 mL milk dilution bottles (Fisher Scientific), autoclaved at 121°C for 15 min (Steris, Mentor, OH, USA), cooled and stored at 4 °C until analysis. All six RUTF formulations were made according to this processing scheme for a total of 24 sample products (Figure 4.1).

2.3 Determination of starch and protein hydrolysis

The degree of starch hydrolysis in the RUTFs was determined using the 3,5-dinitrosalicylic acid method described by Miller (1959) with modifications. For each of the 24 RUTFs (Table 4.1), 2.5 g of liquid product was added to 197.5 mL deionized water and centrifuged at 35,217 x g for 15 min at 20 °C (Beckman Coulter Inc., Brea, CA, USA). One mL of 96 mM 3,5-dinitrosalicylic acid (Sigma Aldrich, St. Louis, MO, USA) was added to two mL of supernatant and boiled at 100 °C for 15 min. The samples were immediately cooled to room temperature on ice and mixed with 9 mL of deionized water. The absorbance was read using a spectrophotometer (ThermoSpectronic, Rochester NY, USA) at 540 nm. A standard curve was made using a 0.2% maltose solution. The amount of sugar released from the starch per mg of solids was calculated and then used to determine the percent of starch hydrolyzed in each RUTF.

The trinitrobenzenesulfonic acid (TNBS) method described by Adler-Nissen (1979) with modifications was used to determine the degree of proteolysis in all RUTF samples. For each RUTF, 2.5 g was mixed with 197.5 mL of 1% sodium dodecyl sulfate (Sigma Aldrich) and centrifuged at 35,217 x g for 15 min at 20 °C. Then, 0.25 mL of the supernatant was added into 2 mL of 0.21 M sodium phosphate buffer (pH 8.20) and 2 ml of 0.1% TNBS (Sigma Aldrich)
solution and samples were incubated for 1 h at 50 °C in a reciprocal shaking water bath (ThermoScientific) with agitation at 80 rpm. Four mL of 0.1 N HCl was added, the samples were cooled for 30 min at room temperature and absorbance was read at 340 nm using a spectrophotometer (Thermospectronic). The standard curve, made using a 5 mM leucine solution, was used to determine the percentage of protein hydrolyzed in the RUTFs.

2.4 Physiochemical analysis of RUTFs

Selected physical properties of all 24 RUTF samples were measured to characterize the products. Viscosity was analyzed using a LV-Brookfield Viscometer (Model LV DV-II+) (Brookfield Engineering Laboratories, Middleboro, MA, USA) by placing 225 ± 5 g of product at 22 °C ± 2 °C in a 500 mL beaker and measuring viscosity at 30 rpm with a LV3 spindle. Color was determined with a Hunter Colorimeter Mini Scan XE (Reston, VA, USA) utilizing the CIE L* a* b* scale. Particle size was analyzed by passing a sample of diluted RUTF (approximately 7 mL in 250 mL deionized water) through a Malvern Mastersizer (Worcestershire, UK). The pH of the RUTFs was measured using an Accumet pH meter (Fisher Scientific) equipped with a pH/ATC gel filled double junction combination electrode, while the moisture content was measured according to the vacuum oven drying method of the AOAC 925.10 (2000).

2.5 RUTF and supplement storage

The effects of storage on microbial and quality characteristics of 12 RUTFs samples containing 0 or 1% bromelain after initial processing and storage at 4 °C for 12 mo were observed. Microbial stability was measured by plating one mL of RUTF on Plate Count Agar (PCA) for total aerobic counts, Potato Dextrose Agar (PDA) for yeast and mold counts and MacConkey agar (BD Difco, Franklin Lakes, NJ, USA) for total coliforms and incubating the plates at room temperature for three days. The RUTFs were plated after initial processing and
after 12 mo of storage at 4 °C. Viscosity, color, pH and moisture content were also measured as described above in section 2.4 after 12 mo of storage at 4 °C.

A vitamin/mineral premix designed specifically to meet the needs of pregnant women, was obtained from Fortitech (Schenectady, NY, USA). The vitamin/mineral premix will be delivered with the RUTFs to provide a nutritionally complete product. The vitamin/mineral premix was filled into opaque, hypromellose capsules (size 0 with a diameter of 7.34 mm; Capsugel, Greenwood, South Carolina, USA). A Capsule Machine filler (Prescott, AZ, USA) was used to make the capsules by separating the cap from the body mechanically, adding the premix and recapping the capsules. The capsules were placed in polypropylene bubble tea straws (12 mm in diameter; 186 mm in height) (Amazon.com, Seattle, WA, USA), heat sealed and stored at 4, 25 or 37 °C for 12 mo. Each month, three capsules were removed from each temperature, weighed (Denver Instrument Company, Göttingen, Germany) and the entire contents of each capsule was emptied into a 50 mL erlenmeyer flask. The vitamin C content (most labile vitamin) of the premix was determined monthly by titration with 2,6-dichloroindophenol based on the AOAC method 967.21 (2000).

2.6 Statistical analysis

Data was analyzed using Statistical Analysis Software (version 9.3; Cary, NC, USA). The degree of starch and protein hydrolysis was examined using regression equations and response surface models. A two-way analysis of variance along with fisher’s least significant difference test was used to determine the significance the physical characteristics and stability of the vitamin/mineral premix and RUTFs at a 95% confidence level. Two independent trials were conducted and for each trial, duplicate samples were analyzed.
3. Results and Discussion

3.1 Starch and Protein hydrolysis

The level of starch hydrolysis for the RUTFs containing rice and barley koji is shown in Figure 4.2A and 4.2B respectively. A response surface model was developed from regression equations to determine the amount of starch hydrolysis that could be achieved at various levels of rice or barley koji. In the model equations, x represents bromelain concentration (%), y equals the koji level (%) and z is the degree of starch hydrolysis (%). RUTFs with rice koji had a significant linear and quadratic component ($p < 0.05$) with the following final model (Figure 4.2A):

\[
z = 36.19 + 2.995x + 2.42y - 0.046y^2 - 0.17xy (R^2=0.97).
\]

The degree of starch hydrolysis for RUTFs containing barley koji also had a significant linear and quadratic component ($p < 0.05$), resulting in the following equation (Figure 4.2B):

\[
z = 37.47 - 22.34x + 2.11y + 20.64x^2 - 0.098y^2 (R^2=0.67).
\]

The degree of proteolysis in the RUTF formulations containing rice and barley koji is displayed in Figure 4.3A and 4.3B. Response surface models were also developed to determine the level of proteolysis that could be achieved at varying levels of bromelain. As described above for starch hydrolysis, x is the bromelain concentration (%), the amount of koji (%) is represented by y and z represents the level of protein hydrolysis (%).

For the degree of proteolysis, the RUTFs containing rice koji had a significant linear component with the following model (Figure 4.3A):

\[
z = 0.83 + 10.33x + 0.011y - 6.72x^2 (R^2=0.88).
\]
RUTF formulations with barley koji also had a significant linear component resulting in the model (Figure 4.3B):

\[ z= 0.16 + 15.74\times + 0.0087\times y – 0.16\times x\times y – 11.17\times y^2 \ (R^2=0.82). \]

Using the response surface models, the levels of koji and bromelain for maximum starch and protein hydrolysis were determined. Maximum starch and protein hydrolysis in RUTFs containing rice koji can be obtained at 21% rice koji and 1% bromelain (Figure 4.2A; Figure 4.3A). The RUTFs with barley koji have a maximum level of hydrolysis at 0.7% bromelain and 6% koji for protein and starch (Figure 4.2B; Figure 4.3B).

The processing scheme developed in the present work showed that simple processing technologies, such as enzymatic hydrolysis, can be utilized to produce nutrient dense RUTFs available in developing countries (Rolle, 1998). The α-amylase source used in the RUTFs was produced using solid substrate fermentation, a low cost, low technology fermentation process (Rolle, 1998, Bechman et al., 2012). Previous work by Pontoh & Low (1995) used α-amylase (165 U in 200 g slurry) to produce a degree of starch hydrolysis of 12-15% in cassava and palm starches in 30-90 min. In the present study, a degree of starch hydrolysis of 16-17% in 1 h (1255 U of α-amylase per 100 g) for RUTFs with rice koji was achieved while the RUTFs containing barley koji had a 11% degradation of starch in 1 h (705 U of α-amylase per 100 g). The degree of starch hydrolysis achieved in 1 h in the current study is consistent with the previous work of Pontoh & Low (1995) in terms of the amount of starch hydrolysis achieved in 1 hr. However, the amount of enzyme used to achieve the hydrolysis is higher in the present work since enzyme hydrolysis was carried out for 4 h to further reduce product viscosity (section 3.2).

The koji used in the present work was found to only have a small amount of proteases (Bechman et al., 2012), therefore bromelain, a proteolytic enzyme found in pineapple juice and
stems, was used (Morton, 1987). Bromelain was selected since pineapples are common in West Africa and commercialization of the enzyme in Mali and the West African region should be feasible (Morton, 1987). The concentration of bromelain was limited to 1% in the RUTFs since higher levels of the protease may have resulted in the formation of bitter peptides during hydrolysis (Morton, 1987).

3.2 Physiochemical properties of RUTFs

The viscosity for all RUTFs is shown in Table 4.2. RUTFs made with rice koji showed a significant decrease \((p < 0.05)\) in viscosity when the level of koji \((\alpha\text{-amylase})\) increased from 7% to 21% (Table 4.2). RUTFs containing barley koji showed a significant difference \((p < 0.05)\), with the viscosity decreasing as the amount of koji rose from 5-15% (Table 4.2). In the presence of bromelain, however, the viscosity of the RUTFs increased. All RUTFs containing 1% bromelain showed a significant difference \((p < 0.05)\) from the other three levels of bromelain: 0, 0.01 or 0.1% for RUTFs containing both rice and barley koji (Table 4.2). The pH, color and particle size were also measured for all RUTFs; however, the data is not shown here as no significant statistical differences were found. The pH of all RUTF formulations had an average of 6.15 (data not shown, \(p > 0.05\)) while the lightness \((L^*)\) value for all products was 49.0 ± 2.2. Particle size ranged from 174-210 µm for RUTFs containing rice koji while the particle size for those comprised of barley koji ranged from 158-222 µm (data not shown).

The increased viscosity seen in the RUTFs with the addition of bromelain may be due to the aggregation of proteins as the products were heated. In cheese making, rennet is used to partially hydrolyze casein, cleaving κ-casein and resulting in partially hydrophobic casein micelles that aggregate forming clumps, then clusters and finally a gel network (Lucey, 2002). Previous research of Prinyawiwatkul, Beuchat, McWatters, & Phillips (1997) with plant proteins...
reported an increase in the viscosity of a cowpea flour and water slurry due to aggregation of proteins during heating. In another study, researchers processed peanut beverages over a temperature range of 85-125 °C and found an increase in viscosity due to changes in protein and carbohydrate structure (Diarra, Nong, & Jie, 2005). The results from the present research are in line with previous work, with the RUTFs containing bromelain thickening after the sterilization process. Another possibility is that the partial proteolysis of the proteins in the RUTFs may have exposed hydrophobic portions, which led to aggregation of the proteins and increased viscosity, similar to the gel casein forms when partially hydrolyzed (Lucey, 2002).

RUTF viscosity is important from a patient standpoint since a thick product is harder to swallow, reducing the nutritional effectiveness (Germain et al., 2006). Previous research by Germain et al. (2006) found the apparent viscosity of thickened nutrition beverages ranged from 615-1480 cP at 8 °C. For all RUTFs in the current research, the viscosity was determined to range from 404-882 cP at 22 °C ± 2 °C (Table 4.2). The RUTFs in present work were measured at 22 °C ± 2 °C since the products will be stored at room temperature due to scarcity of refrigeration in developing countries (Leistner, 1992). While temperature does significantly influence the rheological properties of foods, the RUTFs in this study are in line with the apparent viscosities found in previous work for thickened nutrition beverages, suggesting the RUTFs are suitable for treating even the weakest patients (Gómez-Diaz, & Navaza, 2003; Germain et al., 2006).

Another physical property important to characterizing the RUTFs is particle size. Research has shown that people have the ability to detect particles with a 5 µm diameter or greater (Guinard and Mazzucchelli, 1996). All formulations in the current study have a particle size ranging from 158-222 µm in diameter, which is significantly higher than the detection
diameter, suggesting the RUTFs will have a grainy mouth feel. The particle size and viscosity of the products together may give the product a porridge mouth feel and consistency, which is common in the Malian diet (MacLean & Insoll, 1999).

3.3 RUTFs and supplement storage

The quality attributes measured to determine the effects of storage on the RUTFs including, pH, viscosity and lightness, are displayed in Table 4.3. Viscosity and pH showed significant decreases ($p < 0.05$) between the RUTFs after initial processing and storage for 12 mo at 4 °C (Table 4.3). The lightness measurements were significantly different in the RUTFs ($p < 0.05$) between the two time points as well, with lightness increasing between initial processing and final storage (Table 4.3). For all three measurements, significant differences were also seen between the different RUTF formulations (Table 4.3). All RUTFs were determined to be microbiologically stable since no growth was found after initial processing or storage at 4 °C for 12 mo (data not shown).

The vitamin C content in the vitamin/mineral premix was analyzed monthly to assess the stability of the premix and the delivery method. The vitamin C content showed a change of -0.0003-0.0001 mg at the end of 12 mo for all 3 temperatures. The data is not shown since the values were not significantly different ($p > 0.05$).

The microbial stability and quality attributes of the RUTFs are important aspects of the products. An average pH of 6.15 for the RUTFs (data not shown) classifies the RUTFs as low-acid food products that must be processed to destroy spore-forming microorganisms, specifically *Clostridium botulinum* (Solomon & Lilly, 2001; FDA, 2012). No microbial growth after processing and storage suggests that heating the RUTFs results in a commercially sterile and microbiologically safe product.
Storage conditions vary between developing and developed countries based on climate and access to electricity. Mali has a subtropical to arid climate, with hot-dry, rainy-humid-mild and cool-dry seasons (CIA, 2012). The subtropical climate results in higher room temperatures for shelf storage of the RUTFs as refrigeration is often scarce (Leistner, 1992). Manary (2006) reported that RUTF pastes can be safely stored at ambient tropical conditions for 3-4 months. In the present study, the RUTFs were stored at 4 °C for 1 yr and then assessed for product stability. While storage at 4 °C may not be feasible in Mali, if the product was manufactured and stored in a developed country for shipping at a later date, the product would be refrigerated to prevent any changes in the RUTF composition until shipment.

The vitamin/mineral premix was also found to be stable at all storage temperatures for 12 mo, including 37 °C, suggesting that a nutritionally complete RUTF can be delivered up to 12 mo after processing. The data also suggests that storage in a polypropylene container protected the vitamin/mineral capsules and is a feasible delivery method. The thin polyproylene containers with the capsules will be attached to the bottles of RUTF and the capsules will be added just before consumption.

4. Conclusion

The present research suggests that simple technologies can be utilized to process RUTFs in a chosen country, reducing the cost compared to current commercially available RUTFs. The enzyme levels to produce the RUTFs with maximum pre-digestion of starch and protein were determined to be 21% rice koji with 1% bromelain and 6% barley koji with 0.72% bromelain. Characterizing the product provides baseline information on the product that will allow for standardization of the product, regardless of the processing location. The viscosity ranged from 404-882 cP at 22 °C ± 2 °C for all RUTFs, resulting in drinkable, nutritious semi-liquid foods
that can be used to help prevent malnutrition. Retention of microbiological and quality attributes of the RUTFs and the vitamin/mineral premix are essential to delivering the necessary nutrients needed in a safe product.

Acknowledgements

The authors would like to thank Jerry Davis for providing statistical assistance. This research was made possible through funding provided by the USAID Peanut Collaborative Research Support Program.

References:


Table 4.1. Six formulations processed to determine RUTF product characteristics.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Peanuts</th>
<th>Cowpeas</th>
<th>Millet</th>
<th>Sugar</th>
<th>Rice Koji</th>
<th>Barley Koji</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>38.4</td>
<td>22.2</td>
<td>18.4</td>
<td>14.0</td>
<td>7.0</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>39.0</td>
<td>21.8</td>
<td>11.2</td>
<td>14.0</td>
<td>14.0</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>39.5</td>
<td>21.4</td>
<td>4.1</td>
<td>14.0</td>
<td>21.0</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>38.2</td>
<td>21.9</td>
<td>20.9</td>
<td>14.0</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>E</td>
<td>38.5</td>
<td>21.2</td>
<td>16.3</td>
<td>14.0</td>
<td>-</td>
<td>10.0</td>
</tr>
<tr>
<td>F</td>
<td>38.8</td>
<td>20.5</td>
<td>11.8</td>
<td>14.0</td>
<td>-</td>
<td>15.0</td>
</tr>
</tbody>
</table>
Table 4.2. Viscosity at 30 rpm for processed RUTF formulations containing rice and barley koji based on product type and protease level.

<table>
<thead>
<tr>
<th>Product</th>
<th>Formulations with Rice Koji</th>
<th>Formulations with Barley Koji</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viscosity (cP)</td>
<td>Viscosity (cP)</td>
</tr>
<tr>
<td>A</td>
<td>589a</td>
<td>D 802a</td>
</tr>
<tr>
<td>B</td>
<td>426b</td>
<td>E 701b</td>
</tr>
<tr>
<td>C</td>
<td>422b</td>
<td>F 614c</td>
</tr>
<tr>
<td></td>
<td>404b</td>
<td>0 633b</td>
</tr>
<tr>
<td>0.01%</td>
<td>624b</td>
<td>0.01% 624b</td>
</tr>
<tr>
<td>0%</td>
<td>684b</td>
<td>0% 684b</td>
</tr>
<tr>
<td>1%</td>
<td>882a</td>
<td>1% 882a</td>
</tr>
</tbody>
</table>

Means followed by the same letters in a column are not significantly different ($P > 0.05$).
Table 4.3. pH, viscosity at 30 rpm and lightness of RUTFs at initial processing and after 12 mo of storage at 4°C.

<table>
<thead>
<tr>
<th>Product</th>
<th>pH</th>
<th>Viscosity (cP)</th>
<th>Lightness (L*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 0% bromelain</td>
<td>6.10bc</td>
<td>280e</td>
<td>55.5a</td>
</tr>
<tr>
<td>A 1% bromelain</td>
<td>6.07de</td>
<td>334d</td>
<td>55.7a</td>
</tr>
<tr>
<td>B 0% bromelain</td>
<td>6.12a</td>
<td>179f</td>
<td>55.3a</td>
</tr>
<tr>
<td>B 1% bromelain</td>
<td>6.07e</td>
<td>315d</td>
<td>55.9a</td>
</tr>
<tr>
<td>D 0% bromelain</td>
<td>6.13a</td>
<td>438b</td>
<td>55.6a</td>
</tr>
<tr>
<td>D 1% bromelain</td>
<td>6.12ab</td>
<td>530a</td>
<td>54.3a</td>
</tr>
<tr>
<td>F 0% bromelain</td>
<td>6.09cd</td>
<td>301de</td>
<td>52.1b</td>
</tr>
<tr>
<td>F 1% bromelain</td>
<td>6.04f</td>
<td>401c</td>
<td>52.0a</td>
</tr>
</tbody>
</table>

Storage Time

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Viscosity (cP)</th>
<th>Lightness (L*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>6.16A</td>
<td>638a</td>
<td>49.0b</td>
</tr>
<tr>
<td>Final</td>
<td>6.02B</td>
<td>57b</td>
<td>60.1a</td>
</tr>
</tbody>
</table>

Means followed by the same letter different letters in a column are not significantly different ($P > 0.05$).
Figure Legends:

Figure 4.1- Process flow diagram showing the steps used to make all 6 RUTFs. The diagram begins with the raw ingredients and follows them through until the final heat step of the RUTFs.

Figure 4.2- Starch hydrolysis of RUTF formulations with rice koji (A) and barley koji (B) after 4 h of hydrolysis. The final hydrolysis was determined on all RUTF formulations at all 4 bromelain levels (0.00%, 0.01%, 0.10%, 1.00%). The final amount of starch converted to maltose was calculated and is presented as the percent of starch hydrolyzed in 4 h.

Figure 4.3-Degree of proteolysis for RUTF formulations with rice (A) and barley (B). A total of 3 protease levels (0.01%, 0.10%, 1.00%) along with a control, were added to each RUTF and the total amount of protein hydrolysis was measured. The degree of proteolysis was determined based on the amount of free leucine groups generated and is presented as the percent of protein hydrolyzed by the bromelain in 30 min.
**Fig 4.1. Bechman et al.**

- **Decorticate Cowpeas**

- **Boil on stove separately**
  - Cowpeas
  - Millet

- **Produce Rice Koji and Barley Koji**

- **Pre-roasted Peanuts**

- **Combine: Cowpeas, Millet and Peanuts**
  - Cool to 55 ± 5 °C and add koji

- **Mill all ingredients (and additional water) through colloid mill**

- **Hydrolyze RUTFs at 55°C for 4 hours at 50 rpm**

- **Add bromelain (0, 0.01%, 0.1%, 1.0%)**
  - Hydrolyze 30 min at 55 °C at 50 rpm

- **Boil product on stove for 10 min to inactivate enzymes**

- **Add sugar and salt to RUTFs**
  - Filter through 2mm mesh screen

- **Fill RUTFs in 160 mL bottles and cap**

- **Process the RUTFs at 121°C for 15 min**
Fig 4.2. Bechman et al.

a)

b)
Fig 4.3. Bechman et al.

a)

b)
CHAPTER 5

SURVIVAL OF SIX PROBIOTICS IN CAPSULES WITH RICE AND POTATO
MALTODEXTRIN EXCIPIENTS AT VARIOUS TEMPERATURES

3 Bechman, A, R.D. Phillips and J. Chen. To be submitted to Journal of Food Science
Abstract

The potential benefits of probiotics in the treatment of illnesses and conditions, like malnutrition, have resulted in increased numbers of products entering the market. Since part of the intervention scheme developed in this overall research was to provide probiotics alongside but separate from the RUTFs, it was necessary to determine their viability during storage. The focus of this study was to determine the shelf-life of six probiotic samples, *Lactobacillus rhamnosus* HN001, *Lactobacillus paracasei* Lpc-37, *Lactobacillus plantarum* Lp-115, *Lactobacillus acidophilus* La-14 and *Bifidobacterium lactis* Bl-04, along with a five-strain mixture, stored in hypromellose capsules at 37, 25 and 4 °C in rice or potato maltodextrin for 12 mo to ensure proper probiotic levels are delivered alongside the RUTFs. Monthly, two capsules with each filling material were plated on deMan-Rogosa Sharpe (MRS) agar or MRS with 0.05% L-cysteine hydrochloride and incubated at 37 °C for 72 h under anaerobic conditions. Capsules stored at 4 °C were found to have $10^8$-10$^{11}$ CFU/g remaining after 12 mo for all samples, while samples stored at 37 °C showed the greatest loss of viable cells, with a level of $10^2$-$10^8$ CFU/capsule remaining at 12 mo. During 12 mo of storage, all probiotic samples showed a decrease in cell counts from the beginning to the end of the experiment. Excipient type also influenced probiotic survival, with those stored in rice maltodextrin having higher counts. Based on the results of this study, storage temperature, storage time and excipient type all have an influence on probiotic survival during storage.
Introduction

Probiotics are defined by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO 2001). Studies have shown that probiotics can stabilize gut microflora, stimulate immune system, and provide bioavailable nutrients to the human body (Isolauri 2001; Nagpal and others 2012). The most thoroughly researched probiotics include different species of *Lactobacillus* and *Bifidobacterium* (Fioramonti and others 2003). Strains from these two bacterial genera have been used to treat and reduce the risk of gastrointestinal infections, specifically acute- and antibiotic-associated diarrhea (Hattaka and Saxelin 2008; Nagpal and others 2012).

Another health benefit from probiotics is the potential role probiotics may play in the treatment of malnutrition. Protein energy malnutrition causes a breach in several host defense mechanisms, allowing microbes to invade the intestinal tract, resulting in clinical infections (Cano and others 2002). However, the damaging effects that severe malnutrition can have on the immune system may be reversed through the use of nutrition and other supplements, including probiotics (Cano and others 2002). In a study using treatments with and without *Lactobacillus casei*, along with a standard renutrition diet in malnourished mice, Cano and others (2002) found positive biological effects in the immunosuppressed mice.

With increased consumer awareness on the health benefits of probiotics, including their potential in treating malnutrition, worldwide sales on foods and dietary supplements containing probiotics have increased in recent years, totaling $15.9 billion in 2008 (Yamaguishi and others
Sales on dietary probiotic supplements alone generated $1.5 billion in the same year (Champagne and others 2011).

Manufacturers usually use capsules, tablets or sachets to deliver dietary supplements including probiotics (Fasoli and others 2003). These products are easy to produce, transport and store and can be made shelf-stable for a long period of time. Among the three delivery systems for probiotics, capsules and sachets are preferred as the manufacturing processes are simple with low physical impacts to bacterial cells (Viernstein and others 2005). Although capsules and sachets are both inexpensive delivery vehicles, probiotics packaged in sachets need to be suspended in water or other liquids before consumption (Viernstein and others 2005).

Research has shown that a high percentage of commercial probiotic supplements had lower bacterial counts than what was stated on the product labels (Reid 2005). The false claims may prevent consumers from receiving expected health benefits through probiotic consumption (Knorr 1998). There is currently no established therapeutic level or dose of probiotics for expected health benefits (Reid 2008). However, a daily consumption of $10^8$-$10^9$ CFU per 100 g or mL of products, which is equivalent to $10^6$-$10^7$ CFU/g or mL, has been recommended (Thamaraj and Shah 2004; Angelov and others 2005; Possemiers and others 2010). More recently, higher intakes of $10^8$-$10^{10}$ CFU/day of probiotic organisms have been suggested (Champagne and others 2011).

Several factors can influence the stability of probiotics including storage temperature, storage time, as well as the type of excipient and capsule or sachet used for probiotic delivery (Del Piano and others 2006). The purpose of this research was to determine the stability of five individual probiotic strains as well as a probiotic mixture comprising the five individual strains of probiotics in rice or potato maltodextrin held in hypromellose capsules at 37, 25 or 4 °C.
during a 12 mo storage period in polypropylene bubble tea straws. The straws containing the probiotic capsules will be attached to the bottles of RUTF and will be added just before consumption.

Materials and Methods

Probiotic strains and their delivery systems

Freeze-dried probiotic strains of *Lactobacillus rhamnosus* HN001, *Lactobacillus paracasei* Lpc-37, *Lactobacillus plantarum* Lp-115, *Lactobacillus acidophilus* La-14 and *Bifidobacterium lactis* Bl-04 were generous gifts of Dupont Danisco (Madison, Wisconsin, USA). The five individual strains along with a probiotic mixture comprising an equal amount, by weight, of the five individual strains were used in the present study. Rice maltodextrin (Dupont Danisco) or potato maltodextrin (Ingredion, Bridgewater, NJ, USA) were used as an excipient. Each of the probiotic strains and the five-strain probiotic mixture were mixed separately with each of the two excipients (1:11.6) to create capsule filling materials with a targeted inoculation level of $10^{10-11}$ CFU/g. Clear, hypromellose capsules (size 4 with a diameter of 5.05 mm; Capsugel, Morristown, NJ, USA) were used in the study.

Production of probiotic capsules

The ProFill capsule machine (Torpac Inc, Fairfield, NJ, USA), with a top and a bottom plate, was used to make probiotic capsules. For each probiotic strain/mixture and excipient combination, 100 capsules were produced according to the manufacturer’s recommendations. Briefly, empty capsules were placed into the bottom plate of the capsule-making machine, and
the top plate of the machine was then added. As the two plates were separated, so did the bodies and caps of the capsules. Capsule-filling materials described above were added into the bodies of the capsules, and excess filling materials were removed. Capsule caps were then placed, and assembled capsules with probiotic filling materials were removed from the machine. The capsules were placed in polypropylene bubble tea straws (12 mm in diameter; 186 mm in height) (Amazon, Seattle, WA, USA) (Bruno and Shah 2003) and stored at 37, 25 and 4 °C, respectively. Capsules were sampled monthly for 12 mo, and probiotic populations were determined using the methodology described below.

**Enumeration of probiotic bacteria**

Each probiotic filling material from five individual capsules was pooled and well mixed, and 0.1 g of the resulting sample was added into 9.9 ml of 0.1% peptone water (BD Difco, Franklin, NJ, USA). The samples were then serially diluted in the same solution to determine the initial probiotic counts. Samples containing *L. rhamnosus*, *L. paracasei* and *L. plantarum* were plated on deMan-Rogosa Sharpe (MRS) agar (BD Difco), *L. acidophilus* and *B. lactis* on MRS supplemented with 0.05% L-cysteine hydrochloride (Sigma Aldrich, St. Louis, MO, USA) while the five-strain mixture was plated on both microbiological media. Inoculated plates were incubated at 37 °C for 72 h in anaerobic jars (BBL/Voigt Global Distribution, Lawrence, KS, USA) with anaerobic gas packs (Fisher Scientific, Pittsburgh, PA, USA). Colonies were enumerated following the incubation, and enumeration results were expressed as log CFU/g of probiotic filling materials.

For monthly samplings, two capsules of each filling material were removed from each storage temperature, and the amount of filling material in each capsule was weighed separately.
Exactly 0.1 g of each probiotic sample was placed in 9.9 mL of 0.1% peptone water (BD Difco) before serial dilutions were made. Appropriately diluted probiotic samples were plated in duplicate as described above.

**Statistical analysis**

Data obtained was analyzed using the Statistical Analysis Software (version 9.1; Cary, NC, USA). A two-way analysis of variance along with fisher’s least significant difference test was used to determine the significance of influence of storage temperature, storage time and excipient type on the stability of the probiotic strains/mixture at a confidence level of 95%. Two independent trials were conducted and in each experiment, duplicate samples were analyzed.

**3. Results and Discussion**

Table 5.1 shows the effect of storage time on the stability of six individual probiotic samples used in the present study. The initial inoculation levels of *L. rhamnosus* and *L. paracasei* were $10^{10}$ CFU/g, and those of *L. plantarum*, *L. acidophilus*, *B. lactis* and the probiotic mixture were $10^{11}$ CFU/g (Table 5.1). The counts of *L. plantarum*, *L. acidophilus*, *B. lactis* and the probiotic mixture were not significantly different ($p > 0.05$) from one another at each sampling time point during the entire course of the experiment (Table 5.1). However, the surviving populations of *L. rhamnosus* and *L. paracasei* were significantly different ($p < 0.05$) from the other probiotics and from each other at each individual sampling point except at the beginning of the experiment (Table 5.1). *B. lactis* had the lowest cell population reduction of
3.26 log CFU/g while *L. rhamnosus* had the great cell population reduction of 8.06 log CFU/g during the storage period (Table 5.1).

The average probiotic counts of six individual probiotic strains/mixture stored at 37, 25 and 4 °C were significantly different (*p* < 0.05; Table 5.2). Of the three storage temperatures, all six samples stored at 4 °C had the highest surviving cell population, and those stored at 37 °C had the lowest probiotic cell counts (Table 5.2). Table 5.3 shows the effect of the excipient type on the stability of six individual probiotic samples used in the present study. The cell counts of *L. rhamnosus, L. paracasei, L. acidophilus* and *B. lactis* in rice maltodextrin were significantly higher (*p* < 0.05) than the counts of the same strains carried by potato maltodextrin (Table 5.3).

Individual probiotic strains/mixture followed a similar survival trend in the two excipients; *B. lactis* had the highest cell counts followed by *L. acidophilus*, the five-strain probiotic mixture, *L. plantarum, L. paracasei* and *L. rhamnosus*.

The overall influence of storage temperature, storage time, as well as excipient and strain choice on the survival of the probiotic cultures/mixture is shown in Table 5.4. The average cell counts in samples stored at 37, 25 and 4 °C were significantly different (*p* < 0.05), and the greatest cell viability loss occurred at 37 °C (Table 5.4). The counts of probiotic decreased as the length of storage time increased (Table 5.4). A greater survival was observed with probiotic cells carried by rice maltodextrin (Table 5.4). Cells of *L. rhamnosus* had the poorest survivability followed by those of *L. paracasei* (Table 5.4). The average cell populations of the two bacterial strains were significantly different and were significantly lower than those of the other strains/mixture used in the present study. However, the differences in the cell populations among the other strains were statistically insignificant (Table 5.4).
Stability of the probiotics was influenced by time, temperature and excipient type, suggesting that storage conditions play a critical role in probiotic survival. The observed effects of temperature suggest that lower storage temperatures resulted in higher probiotic survival. One probable reason for the loss of viable cells is due to the difficulty in maintaining bacterial powders at room temperature due to damages that occur to the membranes, proteins and nucleic acids during the drying process (Abe and others 2009a; Yamaguishi and others 2011). These damages, coupled with increased cellular and metabolic activity that lead to exhaustion of stored nutrients in the cell at room temperature, can result in the loss of viable cells in the probiotic capsule (Bruno and Shah 2003). A study with \textit{B. longum} 1941 and \textit{B. longum} 536 found that storage at -18 °C was the best condition for long term storage, while 4 °C reduced viable cell counts and 20 °C led to significant reductions in cell counts (Bruno and Shah 2003). Prasad and others (2003) showed that \textit{L. rhamnosus} HN001, also used in the present work, had a 7.3 log-unit reduction at 30 °C in 14 wk, which is consistent with the present findings, as \textit{L. rhamnosus} had a 3.80 log CFU/g reduction after 1 mo at 37 °C (Table 5.1).

Storage time also has a role in the survival of probiotic cells, as the counts decreased with time for all strains used in the present work. Probiotics are living organisms, and it is inevitable that cell counts will experience a natural decline over time (Del Piano and others 2006). In addition to this decline, processing and storage conditions also affect the length of survival (Del Piano and others 2006). As mentioned above, drying damages bacterial cells, making them more susceptible to death (Abe and others 2009a). A study with \textit{B. longum} BB536, \textit{B. breve} M-16V and \textit{B. infantis} M-63 showed that for all three strains, the reduction in cell counts increased with length of storage (Abe and others 2009b).
Excipient type influenced the probiotic viability in the present work, with the cell counts in rice maltodextrin being significantly different from those in potato maltodextrin (Table 5.4). Maltodextrins are utilized as excipients for probiotics due to a high molecular mass, making them a good bulking agent that has been found to provide a suitable viability for the probiotics (Sollohub and Cal 2010). Certain maltodextrins, such as rice and potato, also function as resistant starches, providing protection to probiotics by withstanding digestion (Fuentes-Zaragoza and others 2011). Differences in the structure of maltodextrins may play a role in their ability to provide stability for the probiotics. While the maltodextrins do provide protection and stability, the materials are hygroscopic. The hygroscopic nature of maltodextrins suggests that moisture may have been absorbed during the study (Sollohub and Cal 2010). This potential change in moisture, and subsequently water activity ($a_w$), could have occurred at different rates in the two excipients, resulting in differences in viability. Kurtmann and others (2009) found that a greater reduction of viable *L. acidophilus* La-5 cells was seen when $a_w$ increased from 0.22 to 0.32. Abe and others (2009) found similar results, with the viability of *B. longum* decreasing with increasing $a_w$. Survival of probiotics during storage is necessary in order to provide health benefits to the consumer. One proposed level of probiotics that must be consumed to achieve the expected health benefits is $10^8$-$10^{10}$ CFU/day (Champagne and others 2011). In the present study, all probiotic samples stored at 4 °C maintained therapeutic levels within the recommended $10^8$-$10^{10}$ CFU/day during 12 mo of storage (Table 5.2). After 12 mo at 25 °C, *L. plantarum*, *L. acidophilus*, *B. lactis* and the five-strain mixture sustained levels that fell within the proposed daily dose (Table 5.2). At 37 °C, however, only *L. acidophilus*, with a count of 8.36 log CFU/g, fell within the suggested daily dose (Table 5.2).
4. Conclusions

Survival of probiotics during storage is essential to provide potential health benefits to treat illnesses and conditions, including malnutrition. In the present work the influence of storage temperature, storage time, excipient type and strain selection were observed to determine their influence on probiotic cell counts. The results of the present study suggest that to provide consumers with $10^8$-$10^{10}$ CFU/day per capsule of viable probiotics, a storage temperature of 4 °C is required. All samples stored at 4 °C had the $10^8$-$10^{11}$ CFU/g viable cells at the end of the 12 mo of storage, while samples stored at 37 °C ranged from $10^2$-$10^8$ CFU/g. *L. rhamnosus* HN001 was found to be the most vulnerable strain in this study, having the greatest loss of viable cells at all temperatures. Storage time was also found to influence probiotic cell counts, with all probiotic samples showing cell loss over 12 mo of storage. Probiotics stored in rice maltodextrin in general had higher remaining log counts compared to those stored in potato maltodextrin. The findings of the study suggest that storage at 4 °C is needed to maintain optimal viability of the tested probiotic strains.

Acknowledgements

This project was made possible through a research grant provided by the United States Agency for International Development. The authors would like to thank Dupont Danisco, Capsugel and Ingredion for their generous donations to this research. The authors would also like to thank Jerry Davis for his assistance with the statistical analysis.
References


Table 5.1. Effects of storage time on the survival (log CFU/g) of probiotics during a 12 mo storage period for all storage temperatures.

<table>
<thead>
<tr>
<th>Storage Time (mo)</th>
<th>L. rhamnosus</th>
<th>L. paracasei</th>
<th>L. plantarum</th>
<th>Five-strain mixture</th>
<th>L. acidophilus</th>
<th>B. lactis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.80A</td>
<td>10.65A</td>
<td>11.55A</td>
<td>11.25A</td>
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<tr>
<td>1</td>
<td>7.00C</td>
<td>8.58B</td>
<td>10.99A</td>
<td>11.20A</td>
<td>11.14A</td>
<td>11.51A</td>
</tr>
<tr>
<td>2</td>
<td>6.62C</td>
<td>8.21B</td>
<td>10.75A</td>
<td>10.59A</td>
<td>10.93A</td>
<td>11.01A</td>
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<td>3</td>
<td>6.27C</td>
<td>8.01B</td>
<td>10.55A</td>
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<td>10.58A</td>
<td>10.59A</td>
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</tr>
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<td>5</td>
<td>4.52C</td>
<td>7.12B</td>
<td>10.13A</td>
<td>9.98A</td>
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<td>10.10A</td>
</tr>
<tr>
<td>6</td>
<td>3.76C</td>
<td>5.98B</td>
<td>9.57A</td>
<td>9.86A</td>
<td>9.93A</td>
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<tr>
<td>7</td>
<td>2.62C</td>
<td>4.65B</td>
<td>8.90A</td>
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<tr>
<td>12</td>
<td>2.74C</td>
<td>4.38B</td>
<td>7.38A</td>
<td>7.81A</td>
<td>8.15A</td>
<td>8.39A</td>
</tr>
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</table>

Means with the same upper case letters in the same row are not significantly different ($p > 0.05$).
Table 5.2. Effects of storage temperature on the survival of probiotics during a 12 mo storage period at 37, 25 and 4°C.

<table>
<thead>
<tr>
<th>Storage Temperature (°C)</th>
<th>L. rhamnosus</th>
<th>L. paracasei</th>
<th>L. plantarum</th>
<th>Five-strain mixture</th>
<th>L. acidophilus</th>
<th>B. lactis</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
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<td>7.66A</td>
<td>7.89A</td>
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<td>7.76A</td>
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<td>6.65C</td>
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<td>10.37AB</td>
<td>10.34AB</td>
<td>10.93A</td>
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<tr>
<td>4</td>
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<td>11.56AB</td>
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Means with the same upper case letters in the same row are not significantly different ($p > 0.05$).
Table 5.3. Effects of rice and potato maltodextrins on the survival of probiotics during a 12 mo storage period.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>L. rhamnosus</th>
<th>L. paracasei</th>
<th>L. plantarum</th>
<th>Five-strain mixture</th>
<th>L. acidophilus</th>
<th>B. lactis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>4.82b</td>
<td>6.22b</td>
<td>9.99a</td>
<td>9.86a</td>
<td>9.89b</td>
<td>9.90b</td>
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<td>Rice</td>
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<td>9.70a</td>
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<td>10.42a</td>
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Means with the same lower case letters in the same column are not significantly different ($p > 0.05$).
Table 5.4. Overall effects of temperature, time, excipient type and probiotic choice on the survival of probiotics during a storage period of 12 mo at 37, 25 and 4°C.

<table>
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</thead>
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<tr>
<td>37</td>
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</tbody>
</table>

<table>
<thead>
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<th>Time</th>
<th>Log CFU/g</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<td>10.07b</td>
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<td>9.68bc</td>
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<table>
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<tr>
<th>Excipient</th>
<th>Log CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>8.45b</td>
</tr>
<tr>
<td>Rice</td>
<td>8.98a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Log CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em></td>
<td>5.28c</td>
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<tr>
<td><em>L. paracasei</em></td>
<td>6.97b</td>
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<tr>
<td><em>L. plantarum</em></td>
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<td>Five strain mixture</td>
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<td><em>L. acidophilus</em></td>
<td>10.09a</td>
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<td><em>B. lactis</em></td>
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Means with the same letter in a column are not significantly different ($p > 0.05$).
CHAPTER 6

DESCRIPTIVE SENSORY EVALUATION OF READY-TO-USE THERAPEUTIC FOODS FOR MALNOURISHED PREGNANT WOMEN

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4 Bechman, A, R Shewfelt, R.D. Phillips and J. Chen. To be submitted to Journal of Food Quality
Abstract

Ready-to-use therapeutic foods (RUTFs) have been shown to be effective in the treatment of malnutrition, which continues to be a major challenge in developing countries. Most RUTFs have been designed for and provided to children who are especially susceptible to malnutrition. However, other groups, particularly women who are pregnant or of child-bearing age, are also prone to malnutrition. In a series of studies, RUTFs containing local plant-based ingredients have been formulated for malnourished pregnant women, specifically those in Mali. The focus of this study was to evaluate the plant-based RUTFs using descriptive sensory analysis to develop a flavor and texture profile for the products. Eleven female graduate students from the University of Georgia were recruited and trained over four sessions to detect seven descriptors: sweetness, bitterness, peanut intensity, beany/cowpea intensity, oxidized, thickness and graininess/grittiness. Twelve RUTFs were evaluated in six testing sessions by the panelists. Of the seven descriptors, peanut intensity and oxidized odor were the two descriptors that showed no significant change ($p > 0.05$). The use of descriptive sensory analysis provided a detailed profile of the flavor and texture profiles of the RUTFs.
Introduction

 Millions of people in the developing world are afflicted by food insecurity, starvation, chronic hunger and malnutrition (Kunyanga et al. 2012). Each year, malnutrition is the direct cause of approximately 300,000 deaths, making it the single most important risk factor for the burden of disease in developing countries (Müller and Krawinkel 2005). Malnutrition continues to be a major cause of morbidity and mortality among the most nutritionally vulnerable groups, specifically infants, children and pregnant women, and continues to be a major focus of research (Müller and Krawinkel 2005; Lartey 2008; Kunyanga et al. 2012).

 A treatment that has proven successful for malnutrition is ready-to-use therapeutic foods (RUTFs), which are nutrient dense foods high in energy and protein that require no additional preparation before consumption (Collins et al. 2006; Manary 2006). The most commonly used RUTF is a paste made of peanuts, powdered milk, sugar, oil sugar and a vitamin/mineral premix (Briend 1999 et al.; Manary 2006). While this treatment has been effective in treating childhood malnutrition, there are limitations to this RUTF formulation. The simple formulation allows the RUTFs to be produced locally in developing countries, however the powdered milk must be imported into most developing countries, greatly increasing the costs (Manary 2006). The World Health Organization (WHO) (2002) recommends the use of locally available crops to provide low-cost nutritional foods with improved quality for specific populations. This is best accomplished by combining local cereals, legumes and starchy roots and tubers in proper proportions to improve protein quality by providing a better balance of essential amino acids and to increase energy (Mensa-Wilmot et al. 2001; Annan et al. 2005).

 Another limitation of current RUTFs is that they were designed for treating childhood malnutrition and little is known about their effectiveness and use in pregnant women.
Malnutrition in pregnant women can have long lasting consequences on both the mother and the child (Lartey 2008). Untreated malnourished mothers are likely to deliver low birth-weight babies who are at a greater risk of impaired mental and physical development and mortality (Victora et al. 2008).

RUTFs for malnourished pregnant women were previously developed by the authors (Bechman et al. Chapter 3) using peanuts, cowpeas, millet and rice or barley, which are local ingredients available in Mali, a West African country with the 2nd highest birth rate, 2nd infant mortality rate and 16th highest maternal mortality rate (CIA 2012) in the world. Once formulated and processed, characterization of the RUTFs was needed to understand the physical and sensory profiles of the RUTFs. The focus of this study was to describe the intensity of seven sensory attributes of 12 RUTF formulations developed from local ingredients for the treatment of malnutrition in pregnant women.

Materials and Methods

RUTF preparation

RUTF formulations based on ingredients commonly found in Mali were developed using nutrient optimizing, low costing formulation software (Creative Formulation Concepts, LLC, Annapolis, MD, U.S.A.) (Bechman et al. Chapter 3). Six base formulations containing peanuts, cowpeas, millet, sugar and rice koji or barley koji were chosen for processing (Table 6.1). Medium grain white rice and pearled barley were obtained locally (Sevananda Natural Foods Market, Atlanta, GA, U.S.A.), and fermented with Aspergillus oryzae to produce koji, a source of amylolytic and proteolytic enzymes (Bechman et al. 2012). Decorticated cowpeas (Inland Empire Foods, Riverside, CA, U.S.A.) and millet flour (Bob’s Red Mill, Milwaukie, OR, U.S.A.) were boiled in tap water (1:10) and then mixed together, along with pre-roasted peanuts.
(American Blanching Company, Fitzgerald, GA, U.S.A.). Once cooled to 55°C ± 5C, rice koji (7,
14, or 21% of total solids) or barley koji (5, 10, or 15% of total solids) was added. Based on the
formulations (Table 6.1), 100 g of RUTFs containing the rice koji (dry weight) had 418, 837 or
1255 units (expressed as mg maltose released/g koji solids/min) of α-amylases while the same
amount of RUTFs with barley koji (dry weight) had 587, 1175 and 1762 units of α-amylases
(Bechman et al. 2012). Once the koji had been added, additional water (71.67-244.60 mL) was
added, and the diluted mixtures were passed two times through a colloid mill (Morehouse
Industries, Los Angeles, CA, U.S.A.). The water added to each formulation was determined by
subtracting the water used for boiling the cowpeas and millet flour from the total batch size of a
RUTF intended to be prepared. The milled product was divided into two 8 quart stainless steel
containers (Crate and Barrel, Northbrook, IL, U.S.A.), covered with glass lids and incubated at
55°C in a reciprocal shaking water bath at 50 rpm (ThermoScientific, Marietta, OH, U.S.A.) for 4
h. Bromelain, equivalent to 0% or 1% of total predicted protein with an activity of 6 Gelatin
Digesting Units/g (Kalyx, Camden, NY, U.S.A.), was added to the containers of product, and
incubated for 30 min at 55°C with a shaking speed of 50 rpm. The products were boiled on a
stove (Amana, Benton Harbor, MI, U.S.A) for 10 min to denature proteases. Sugar (Table 6.1)
and salt (0.1% dry weight basis) were blended into the product which was filtered through a 2
mm mesh screen (Fisher Scientific, Pittsburgh, PA, U.S.A.), filled into pint-size (16 oz) Ball®
canning jars (Wal-Mart, Griffin, GA, U.S.A.) and sterilized for 15 min at 121°C (Yamato
Scientific America Inc., Santa Clara, CA, U.S.A). The two different koji types at three levels in
the presence or absence of protease resulted in a total of 12 RUTF formulations (Table 6.1).
Sensory evaluation

Eleven females at the University of Georgia of childbearing age, 15–49, as defined by the World Health Organization (2012), were recruited to form a trained panel to evaluate the RUTFs. Descriptors for the RUTFs were determined by a small preliminary panel consisting of the researchers and graduate students. Seven descriptors were chosen for the evaluation of the RUTFs: sweetness, bitterness, peanut intensity, beany/pea intensity, oxidized odor, thickness and graininess/grittiness (Table 6.2). The panelists were trained over four 1 h sessions. Two 1 h training sessions were conducted to determine the reference standards and set their scale values for each descriptor (Table 6.2). The additional two sessions were used to familiarize the panel with the ballot (Appendix B) and to train them to consistently evaluate and score a RUTF sample based on the 7 descriptors. Samples were analyzed using a 150 mm unstructured intensity scale (0 mm = none, 150 mm = extreme/intense) for each descriptor (Meilgaard et al. 2006; Felland and Koehler, 1996). Each panelist completed one ballot (Appendix B) per RUTF sample using a ruler to mark the intensity for each descriptor. Descriptive sensory analysis was conducted on 12 RUTF products (Table 6.1) over six testing sessions. In each session, four RUTF samples (1 oz each) were presented simultaneously in 2 oz plastic soufflé cups with lids (Thorton Brothers, Athens, GA U.S.A.). Each RUTF sample was given a randomly generated three digit code and the order of sample presentation was also randomly assigned. Panelists evaluated the RUTFs in individual booths with normal lighting. All 12 RUTFs were presented in duplicate during the six sessions. Two test sessions were conducted per week for a total of three testing weeks. For each sample, a new jar of RUTF was opened, stirred, poured into sample cups and brought to room temperature before the samples were presented to the panelists. All panelists were given tap water filtered through a Brita pitcher (Brita, Oakland, CA, U.S.A.) in 6 oz styrofoam cups
(Thornton Brothers), all reference standards used during the training sessions and saltine crackers with unsalted tops (Wal-Mart, Athens, GA, U.S.A) to cleanse their palate between samples. For all samples, one ballot per sample was completed with the same design described above being used. This sensory test was approved by the University of Georgia Institutional Review Board (project number: 2012-10548-0).

Statistical Analysis

Data obtained was analyzed using Statistical Analysis Software (version 9.3 Cary, NC, U.S.A.). A two-way analysis of variance (ANOVA), along with Fisher’s least significant difference test was used to determine the significant difference in the RUTF samples for each descriptor based on a confidence level of 95%. Each descriptor was analyzed by grain type and protease level.

Results and Discussion

Table 6.3 shows the five descriptors that exhibited significant differences ($p < 0.05$) as detected by the panelists: sweetness, bitterness, beany/pea, thickness and graininess/grittiness. For the sweetness descriptor, a significant difference ($p < 0.05$) was seen in the RUTFs containing rice koji with 0% bromelain as the level of koji increased from 7% to 21% (Table 6.3). The panelists detected a decrease in bitterness for the RUTFs with 1% bromelain and rice koji that was significantly different ($p < 0.05$) when the amount of koji changed from 7 to 21% (Table 6.3). In the beany/pea descriptor no significant difference ($p >0.05$) at 0% bromelain was seen in the RUTFs containing either rice or barley koji (Table 6.3). The only significant change for the beany/pea descriptor was seen in RUTFs comprised of 15% barley koji with 1% bromelain compared to the 5 and 10% RUTFs with barley koji at the same bromelain level (Table 6.3). The thickness descriptor showed a significant change in RUTFs containing both rice
and barley koji at 0 and 1% bromelain. RUTFs containing rice koji and 0% bromelain had a significant decrease ($p < 0.05$) in thickness, with the 21% koji level being significantly different from the other two levels while RUTFs with 1% bromelain and rice koji showed a decrease at each increase in koji (Table 6.3). The RUTFs with barley koji showed a significant decrease ($p < 0.05$) in thickness at each increasing level of koji at 0% bromelain, but in the presence of 1% bromelain, the RUTFs with 5% barley koji were significantly different from the other two levels (Table 6.3). Differences in graininess/grittiness were significant with the RUTF with 14% rice koji and 1% bromelain being different from those with 7 and 21% rice koji at the same level of bromelain (Table 6.3). For the RUTFs with barley koji, a significant difference ($p < 0.05$) was detected in the RUTF with 5% koji and 1% bromelain compared to the remaining levels of koji (Table 6.3).

Differences in the flavor and texture profile of the 12 RUTFs were detected by the panelists for five of the seven descriptors. The panelists detected more changes in the thickness of the RUTFs compared to the other descriptors (Table 6.3). Changes in RUTF thickness was expected due to the use of $\alpha$-amylases, provided by the rice and barley koji, to degrade the starch granules in the RUTFs and decrease RUTF thickness (Helland et al. 2002; Bechman et al. 2012). Previous research with rice porridge weaning foods made from amylase rich flours showed that viscosity of the weaning foods decreased as much as 98%, depending on the quantity of amylase rich flour used (Gopaldas et al. 1986; Wahed et al. 1994). In the present work, the RUTFs were previously analyzed to determine viscosity (Bechman et al. Chapter 4). The RUTFs containing 7% rice koji with 0% bromelain, had a viscosity of 506 cP at 30 rpm while those with 14-21% koji had viscosities of ~352 cP (Bechman et al. Chapter 4). When 1% bromelain was added to the RUTFs with rice koji, the viscosity increased, with the RUTF with 7% rice koji having a
viscosity of 610 cP while the lowest viscosity was 568 cP for 21% rice koji (Bechman et al. Chapter 4). However, the RUTFs comprised of barley koji had higher viscosities overall with or without bromelain. In the absence of bromelain, the RUTFs with 5% barley koji had a viscosity of 776 cP compared to 570 cP with 15% barley koji RUTFs (Bechman et al. Chapter 4). The addition of 1% bromelain, however, resulted in a viscosity of 971 and 744 cP for 5% and 15% barley koji (Bechman et al. Chapter 4). The previously determined viscosities are in agreement with the changes detected in the sensory analysis. In general, the RUTFs with bromelain had higher ratings by the panelists (Table 6.3) which are in agreement with the higher viscosities (Bechman et al. Chapter 4). Sensory analysis also showed that RUTFs comprised of rice koji were thinner compared to the RUTFs with barley koji with or without bromelain (Table 6.3), which is also in agreement with the viscosities determined for the products (Bechman et al. Chapter 4).

Enzymes (α-amylase and bromelain) were used in the RUTFs to improve the nutrient digestibility while reducing the viscosity to produce semi-liquid RUTFs. However, these enzymes also impact the flavor profile of the RUTFs. While degrading the starch, α-amylases convert the starch into simple sugars, specifically maltose, increasing sweetness in the RUTFs (Helland et al. 2002). A study by Helland et al. (2002) used germinated corn flour as an enzyme source to reduce the viscosity of corn porridge and found the levels of maltose and glucose increased in the presence of enzymes. In the current work, the sweetness of the RUTFs with rice koji and 0% bromelain increased as the enzyme level (koji) rose (Table 6.3). Bechman et al. (Bechman et al. Chapter 4) determined that the degree of starch hydrolyzed in the RUTFs with 0% bromelain was 50%, 60% and 67% at rice koji levels of 7, 14 and 21%, respectively.
This increase in starch hydrolysis seen in the RUTFs is in line with the increase in sweetness detected by the panelists in the RUTFs (Table 6.3).

However, a significant difference was not seen in the sweetness of the other products, despite similar levels of starch hydrolysis (Bechman et al. Chapter 4). One reason for no significant difference ($p > 0.05$) in sweetness in the RUTFs containing 1% bromelain may be due to the presence of bitter peptides. Proteases, like bromelain, have been found to produce bitter peptides during protein hydrolysis by exposing hydrophobic amino acids (Saha and Hayashi 2001). Bechman et al. (Chapter 4) found a degree of protein degradation ranging from 2.23-4.86% for the RUTFs containing 1% bromelain, which could have resulted in a bitter taste. Bitterness and sweetness have been found to mutually suppress each other (Keast and Breslin 2002), which could have affected the panelists ability to detect a difference in sweetness. Previous research has shown that sucrose and caffeine were easier to detect in a liquid solution, while being somewhat difficult to detect in a foam, and most difficult to detect in a gel type medium, implying that thickening agents create a detection threshold (Arabie and Moskowitz 1971). The viscosity of the RUTFs in the present work ranges from 352-971 cP (Bechman et al. Chapter 4), which suggests a significant change was not seen in the RUTFs with barley koji due to a perceived difference in thickness.

The thickness of the product, along with the mutual expression seen between sweetness and bitterness, may also explain why bitterness was only perceived in the RUTFs containing rice koji with 1% bromelain. Breslin and Beuchamp (1995) looked at the interactions of different salts on the effect of bitterness in solutions and found that salt suppressed the bitter flavor. The RUTFs in the present study contained 0.1% salt (sodium chloride), which could help suppress the bitter flavor. The influence of the salt, along with the viscosity of the products, might work
together to increase the threshold level of detection while suppressing the bitterness, resulting in bitterness only being detected in the RUTFs comprised of rice koji with 1% bromelain.

The beany/pea and graininess/grittiness descriptors appeared to be more challenging for the panelists to differentiate and agree on in the RUTFs. These RUTFs are a complex food matrix, made of four different plant ingredients, along with sugar and salt. The mixture of ingredients could have affected the detection threshold of the various descriptors due to the interaction of compounds (Drake and Civille, 2002). Marshall et al. (2006) conducted a study looking at mixtures of stimuli containing 1-6 components and found that in solutions containing more than 4 components, panelists could only identify up to 2 of the stimuli. Due to the complexity of the RUTFs tested in this work, it is possible that the panelists were not able to distinguish the beany/pea attribute from the other flavor compounds and ingredients.

The complexity of the product may have had an impact on panelists’ ability to determine the level of graininess/grittiness in the RUTFs. Significant changes in the graininess/grittiness were only seen in the RUTFs containing 1% bromelain, with both types of koji (Table 6.3). Along with the complex nature of the RUTFs, taste and texture preferences vary from one person to the next (Keast and Breslin 2002). Even with the same individual, a perceived intensity of a descriptor can change depending on the food and drink consumed before testing (Keast and Breslin 2002). It is possible that the changes seen in the graininess/grittiness descriptor are due to variation in how the descriptor was interpreted among the panelists.

**Conclusions**

RUTFs using local ingredients to help treat malnutrition in pregnant women have been developed using least-cost computer formulation software. In order to consistently produce the RUTFs, full characterization of the products, including descriptive sensory analysis is needed.
The sweetness, bitterness, thickness, beany/pea and graininess/grittiness descriptors were found to have significant differences \((p < 0.05)\), suggesting they have an influence on the overall flavor and texture profile of the RUTFs. The thickness of the RUTFs decreased as the amount of \(\alpha\)-amylase increased, suggesting that the enzymes did reduce the overall viscosity of the RUTFs. The use of descriptive sensory analysis provided a detailed profile of the flavor and texture profiles of the RUTFs.

**Acknowledgements**

This project was made possible through a research grant provided by the United States Agency for International Development. The authors would also like to thank Jerry Davis for his assistance with statistical analysis.

**References:**


Table 6.1. Composition of RUTF formulations developed and processed for sensory evaluation.

<table>
<thead>
<tr>
<th>RUTFs</th>
<th>Peanuts</th>
<th>Cowpeas</th>
<th>Millet</th>
<th>Rice</th>
<th>Barley</th>
<th>Sugar</th>
<th>Bromelain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>38.41</td>
<td>22.23</td>
<td>18.36</td>
<td>7.00</td>
<td>0.00</td>
<td>14.00</td>
<td>0.00</td>
</tr>
<tr>
<td>A2</td>
<td>38.41</td>
<td>22.23</td>
<td>18.36</td>
<td>7.00</td>
<td>0.00</td>
<td>14.00</td>
<td>1.00</td>
</tr>
<tr>
<td>B1</td>
<td>38.95</td>
<td>21.81</td>
<td>11.24</td>
<td>14.00</td>
<td>0.00</td>
<td>14.00</td>
<td>0.00</td>
</tr>
<tr>
<td>B2</td>
<td>38.95</td>
<td>21.81</td>
<td>11.24</td>
<td>14.00</td>
<td>0.00</td>
<td>14.00</td>
<td>1.00</td>
</tr>
<tr>
<td>C1</td>
<td>39.49</td>
<td>21.40</td>
<td>4.11</td>
<td>21.00</td>
<td>0.00</td>
<td>14.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C2</td>
<td>39.49</td>
<td>21.40</td>
<td>4.11</td>
<td>21.00</td>
<td>0.00</td>
<td>14.00</td>
<td>1.00</td>
</tr>
<tr>
<td>D1</td>
<td>38.17</td>
<td>21.92</td>
<td>20.91</td>
<td>0.00</td>
<td>5.00</td>
<td>14.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D2</td>
<td>38.17</td>
<td>21.92</td>
<td>20.91</td>
<td>0.00</td>
<td>5.00</td>
<td>14.00</td>
<td>1.00</td>
</tr>
<tr>
<td>E1</td>
<td>38.46</td>
<td>21.20</td>
<td>16.34</td>
<td>0.00</td>
<td>10.00</td>
<td>14.00</td>
<td>0.00</td>
</tr>
<tr>
<td>E2</td>
<td>38.46</td>
<td>21.20</td>
<td>16.34</td>
<td>0.00</td>
<td>10.00</td>
<td>14.00</td>
<td>1.00</td>
</tr>
<tr>
<td>F1</td>
<td>38.75</td>
<td>20.28</td>
<td>11.77</td>
<td>0.00</td>
<td>15.00</td>
<td>14.00</td>
<td>0.00</td>
</tr>
<tr>
<td>F2</td>
<td>38.75</td>
<td>20.28</td>
<td>11.77</td>
<td>0.00</td>
<td>15.00</td>
<td>14.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Table 6.2. Descriptors and standards used for the sensory evaluation of the RUTFs.

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Definition</th>
<th>References (mm)</th>
<th>Rating Scale (mm)</th>
</tr>
</thead>
</table>
| Sweetness*          | Total sweetness associated with sucrose solutions | 2.0% sucrose solution= 20  
5.0% sucrose solution= 50  
10.0% sucrose solution= 100 | No sweetness = 0  
Extremely sweet = 150 |
| Bitterness*         | Basic bitter taste associated with caffeine solutions | 0.05% caffeine= 20  
0.08% caffeine= 50  
0.15% caffeine= 100 | No bitterness = 0  
Extremely bitter= 150 |
| Peanut*             | Intensity of roasted peanut flavor | Planters Cocktail Peanuts = 70 | No intense peanut flavor = 0  
Intense peanut flavor = 150 |
| Beany/Pea           | Intensity of beany/pea flavor | Bush’s Blackeye Peas = 60  
Bush’s Garbonazo Chick Peas = 100 | No intense beany/pea flavor = 0  
Intense beany/pea flavor= 150 |
| Oxidized*           | Odor associated with aged oils and fat | Wesson vegetable oil (microwaved)= 80 | No oxidation = 0  
Extremely oxidized = 150 |
| Thickness           | Measure of viscosity within the mouth | 50% Karo syrup solution = 50  
Karo syrup = 100 | Thin = 0  
Thick = 150 |
| Graininess/Grittiness | Amount of small particles of sample remaining in the mouth after swallowing | Naked Juice Red Machine = 30  
Naked Juice Mango Veggie = 55  
Naked Juice Protein Zone = 110 | No graininess/grittiness = 0.0  
Extremely grainy/gritty = 150.0 |

*aDefinitions were discussed with the panelists and provided orally but were not written down during the training or testing sessions.

*bAll materials for the reference standards were purchased at Wal-Mart and Kroger in Athens, GA, U.S.A.

*cReference standards were made according to Meilgaard et al. (2006).

*dReference standard was made according to Vázquez-Araújo et al. (2012).
Table 6.3. Comparison of the 5 descriptors showing a significant difference analyzed on grain type (rice vs. barley) and the amount of bromelain (0% vs. 1%).

<table>
<thead>
<tr>
<th>Product</th>
<th>Sweetness</th>
<th>Bitterness</th>
<th>Beany/Pea</th>
<th>Thickness</th>
<th>Graininess/Grittiness</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rice Formulations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7% Rice Koji</td>
<td>38b</td>
<td>36a</td>
<td>20a</td>
<td>28a</td>
<td>55a</td>
</tr>
<tr>
<td>14% Rice Koji</td>
<td>43ab</td>
<td>38a</td>
<td>19a</td>
<td>25ab</td>
<td>52a</td>
</tr>
<tr>
<td>21% Rice Koji</td>
<td>45a</td>
<td>40a</td>
<td>16a</td>
<td>19b</td>
<td>49a</td>
</tr>
<tr>
<td><strong>Barley Formulations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% Barley Koji</td>
<td>38a</td>
<td>34a</td>
<td>17a</td>
<td>20a</td>
<td>50a</td>
</tr>
<tr>
<td>10% Barley Koji</td>
<td>39a</td>
<td>32a</td>
<td>20a</td>
<td>23a</td>
<td>53a</td>
</tr>
<tr>
<td>15% Barley Koji</td>
<td>38a</td>
<td>35a</td>
<td>24a</td>
<td>25a</td>
<td>54a</td>
</tr>
</tbody>
</table>

Means (n = 22) followed by the same letter in the same column within the same type of formulation are not significantly different (p > 0.05).
CHAPTER 7

CONCLUSION

Malnutrition is a serious condition with short and long term consequences affecting not only the individual but the entire household. Treatment of malnutrition with ready-to-use therapeutic foods (RUTFs) containing peanuts, powdered milk, sugar, oil and vitamin/minerals has been proven successful in children. Formulation and development of RUTFs for other nutritionally vulnerable using plant based ingredients is needed to provide appropriate treatment options. In order to focus the research, the present study concentrated on developing RUTFs for malnourished pregnant women in Mali using computer software to develop low-cost RUTFs using local plant ingredients.

Six base RUTF formulations were developed using formulation computer software that contained peanuts, cowpeas, millet and rice or barley in the form of koji to serve as both an ingredient and an enzyme source. Once formulated, simple technologies including roasting, decortication, boiling, milling, enzymatic hydrolysis and heat sterilization, were utilized to process the RUTFs. The processed RUTFs were analyzed to determine the nutrient content of the RUTFs to compare to the software predicted values. The results of this analysis showed that the actual values were similar to software predicted values, with energy having the largest difference, being 11.10-19.70% higher than predicted. The differences seen in predicted and actual nutrient contents is most likely due to the differences between reference and actual nutrient values of the ingredients used in the study. The amount of RUTF that must be consumed to provide the nutrients required in one day for pregnant women varies depending on
both the nutrient and the formulation, but using the limiting nutrients of energy and lysine, approximately 2,620-3,002 g must be consumed.

Enzymatic hydrolysis was used during processing to break down starch and protein, making the RUTFs easier for malnourished patients to digest and also reducing the viscosity. Surface response modeling was used to determine a maximum koji and bromelain level. The results found that for RUTFs containing rice koji, a level of 21% koji and 1% bromelain would produce the maximum hydrolysis results desired. In the RUTFs with barley koji, an amount of 6% barley koji and 0.72% bromelain would provide maximum hydrolysis. Viscosity of the RUTFs was analyzed as well, with the results showing that at 30 rpm, the viscosity decreased in all formulations decreased as the levels of koji increased from 7-21% and 5-15% for formulations with rice and barley koji, respectively. In the presence of bromelain, however, the viscosity increased as the level of bromelain increased from 0-1% in all RUTFs.

After processing, the RUTFs were then evaluated through descriptive sensory analysis to develop a flavor profile and a better understanding of the flavor interactions of the ingredients. The panelists evaluated twelve products on seven descriptors including sweetness, bitterness, peanut intensity, beany/pea intensity, oxidized odor, thickness and graininess/grittiness. From the sensory testing, it was determined that significant differences (p <0.05) were observed by the panelists in sweetness, bitterness, beany/pea intensity, thickness and graininess/grittiness. In all RUTFs, with or without bromelain, the panelists detected a decrease in thickness as the level of koji increased in the products. The differences detected in sweetness and bitterness by the panelists was seen only in the products containing rice koji, which is thought to be due to the complexity of the products along with different flavors in the RUTFs due to the rice and barley koji.
Malnutrition affects the whole body in an affected person, including the intestinal tract by affecting nutrient absorption and immune response. Research has been conducted looking at the effects of probiotics on malnutrition, with positive outcomes being found, including faster recovery of the villi of the intestinal tract and increased levels of IgA+ cells, which play a key role in host immunity. However, probiotics are living organisms that show natural decay over time which affects the survival of probiotics during storage. The results of the present research found that all six probiotics stored at 4 °C had the best survival compared to 25 and 37 °C. All six probiotic samples showed decrease from the beginning of the experiment to the end, with *B. lactis* having the highest level of remaining cells and *L. rhamnosus* having the lowest surviving population.

While the focus of this research was to develop RUTF formulations using plant based ingredients to treat malnourished pregnant women in Mali, the strategy utilized in this research can be applied to other countries in Africa. The results of the present work suggest that RUTFs can be formulated and processed employing local plant based ingredients and simple technologies. Regardless of the country being targeted, the basic steps and methodologies developed in this research can be transferred and used to develop new RUTFs. Computer formulation software allows for the formulations to be developed quickly based on minimizing cost while meeting the nutrient requirements by entering the ingredients available into the software database. The processing scheme may vary depending on technology available, however the basic steps, including enzymatic hydrolysis and heat sterilization, will still be necessary in some form. Enzymatic hydrolysis reduces the overall viscosity of the product making it easier to consume while heat sterilization must be included to render the RUTFs
microbiologically safe and shelf stable. The flexibility of the approach described here allows the RUTF formulation to be tailored to fit the needs and culture of a given country.

The present work demonstrated the development, processing and characterization of the RUTFs, but further research is still needed, including clinical trials to determine the efficacy of these RUTF formulations in malnourished pregnant women to fully realize the potential of these RUTFs. Additional research is also needed to expand this approach to other target countries.
APPENDIX A

CHANGES IN SELECTED PHYSICAL PROPERTY AND ENZYME ACTIVITY OF RICE AND BARLEY KOJI DURING FERMENTATION AND STORAGE

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Abstract: Koji are solid-state fermentation products made by inoculating steamed grains with the spores of fungi, particularly *Aspergillus* spp. This research was undertaken to identify the fermentation and storage conditions optimal for the production and maintenance of selected hydrolytic enzymes, such as α-amylase and protease, in koji. Steamed rice and barley were inoculated with 2 X 10^{11} *Aspergillus oryzae* spores per kg of grains and fermented for 118 h in a growth chamber at 28-32°C with controlled relative humidities. Samples were drawn periodically during fermentation and storage at -20, 4, or 32 °C, and α-amylase and protease activity, mold counts, a_{w}, moisture contents, and pH of collected samples were determined. It was observed that the a_{w}, moisture contents, and pH of the koji were influenced by the duration of fermentation and temperature of storage. The α-amylase activity of both koji increased as the populations of *A. oryzae* increased during the exponential growth phase. The enzyme activity of barley koji was significantly higher than that of rice koji, reaching a peak activity of 211.87 or 116.57 U at 46 and 58 h, respectively, into the fermentation process. The enzyme activity in both products started to decrease once the mold culture entered the stationary growth phase. The protease activities of both koji were low and remained relatively stable during fermentation and storage. These results suggest that rice and barley koji can be used as sources of α-amylase and desired enzyme activity can be achieved by controlling the fermentation and storage conditions.

Keywords: *Aspergillus oryzae*, koji, fermentation, α-amylase, protease
**Introduction**

Amylases and proteases are two classes of enzymes that are used in a variety of fields: from detergent manufacturing to food processing. In the food industry, these enzymes are used to break down starches and proteins while reducing the viscosity of foods. Although amylases and proteases are found in plants and animals, commercial enzymes are often produced using bacteria or molds (Chutmanop and others 2008) through solid state fermentation which is designed to use natural microbial process to produce enzymes in a controlled environment (Couto and Sanroman 2006).

*Aspergillus oryzae* is a filamentous fungus that is known for its ability to produce many hydrolytic enzymes (Liang and others 2009). Several traditional Asian food products, such as soy sauce, sake (rice wine), shochu (spirits), and miso are made through solid-state fermentation using *A. oryzae* (Abe and others 2006). Steamed grains, such as soybeans, rice, wheat, and barley have been used as the substrate for *A. oryzae* to produce desired final products (Giri and others 2011). The starting substrates and mold strains selected for the fermentation influence the profiles and concentrations of the hydrolytic enzymes, and subsequently the flavors of the final products (Giri and others 2009).

Enzymes in fermented grains, known as koji, have been used to hydrolyze starches or proteins in the production of squid miso (Giri and others 2011) and fermented black beans (Lee and others 2008). Although previous research has found beneficial hydrolytic enzymes in koji, fermentation conditions including temperature, time, and humidity that are optimal for hydrolytic enzyme production have not been adequately researched. Furthermore, the activities of the enzymes under different storage conditions have not been thoroughly investigated.
The objectives of this research include: 1) to determine the fermentation conditions that are optimal for the production of amylases and proteases in koji made with two different starting materials, rice and barley, 2) to determine the stability of amylases and proteases in rice and barley koji stored at 3 different temperatures, 32, 4, and -20 °C.

**Materials and Methods**

**Koji production**

Polished white rice and pearled barley were obtained from a local cooperative market (Sevananda, Atlanta, GA, U.S.A.). The rice (4 kg) was soaked in 8 L of distilled water for 16 h while the barley (4 kg) was soaked in 10 L of water for 2 h, both at room temperature. Soaked grains were drained using a self made mesh wire tray (18 x 18 x 1.75 in) and then steamed in a kettle (Lee Metal Products, Phillipsburg, PA, U.S.A) for 50 min at 40 to 45 psi (Fujita and others 2003). Once cooled to 30 to 32 °C, the grains were inoculated with the spores of *A. oryzae* W20 at 2 g/kg (1 x 10\(^{11}\) spores/g; Higuchi Matsunosuke Shoten Co, LTD, Osaka, Japan). The inoculated grains were transferred onto the mesh wire trays lined with a steamed cloth, at approximately 1 kg per tray, and covered with another steamed cloth. The trays were placed into a growth chamber at 30 °C and a 98% relative humidity. A k-type thermocouple (Omega, Stamford, CT, U.S.A.) was inserted into each tray to record product temperature. The chamber temperature and humidity were monitored through a control sensor (Omega, Stamford, CT, U.S.A.) that was run by a HP data acquisition/switch unit (Hewlett Packard, Palo Alto, CA, U.S.A.) controlled by a computer. After 24 h of fermentation, the fermented rice was hand mixed for approximately 3 min (Machida and others 2008) before being returned to the chamber at 30 °C with a 98% relative humidity with the humidity dropped 2% every 4 h. The barley koji
was mixed after 20 h of fermentation and returned to the same growth chamber with the humidity dropped every 3.43 h.

**Sample collection during fermentation**

Steamed grains described above were fermented for a total of 118 h. The koji were hand mixed for 3 min every 12 h. After each mixing, 30 g of each type of koji was collected, placed in sterile sampling bags (Fisher Scientific, Pittsburgh, PA, U.S.A), and stored at -20 °C prior to analysis.

**Sample collection during storage**

Grains fermented for 44 h were used in the storage study. Exactly 30 g of each type of koji was drawn and placed in sterile sampling bags (Fisher Scientific). The samples were stored at 32, 4, or -20 °C, and analyses were performed at different intervals: the 32 °C samples were assayed daily for 9 d, while those stored at 4 °C were sampled bi-weekly for 12 wk. The samples at -20 °C were analyzed monthly for 12 mo.

**Enzyme extraction and physical properties of rice and barley koji**

The moisture contents of collected samples were measured (32.1.36; AOAC 2000) in order to estimate the amounts of solids or the dry weights of koji. A Decagon Pawkit water activity meter (Pullman, WA, U.S.A.) was used to measure the water activities ($a_w$). Enzymes in the koji samples were extracted using a method recommended by Megazyme (2004). Exactly 15 g of each type of koji was mixed with 150 mL of deionized water and blended for 30 and 45 s, respectively using a food chopper (Black and Decker, New Britain, CT, U.S.A.). After the pH of
the product slurry was measured with an Accumet pH meter (Fisher Scientific, Pittsburgh, PA, U.S.A.), the homogenate was placed in a reciprocal shaking water bath (ThermoScientific, Marietta, OH, U.S.A.) at 30 °C for 30 min with agitation at 80 rpm (Chutmanop and others 2008). The homogenate was then centrifuged at 15,652 x g for 12 min at 4 °C. The α-amylase and protease activities in the collected supernatants were determined using the procedures described below.

**α-amylase activities of koji**

The α-amylase activities of koji were determined using the 3, 5-dinitrosalicylic acid method as described by Miller (1959) with modifications. The supernatants described above were serially diluted in deionized water, and 1 mL of each diluted supernatant was incubated with 1 mL of 1% soluble starch solution, in 20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.9), for 10 min at 55 °C with agitation at 80 rpm. Then 1 mL of 96 mM 3, 5-dinitrosalicylic acid (Sigma Aldrich, St. Louis, MO, U.S.A.) was added, and the samples were boiled at 100 °C for 15 min. The heated samples were immediately cooled on ice to room temperature, and mixed with 9 mL of deionized water. The absorbance was read using a spectrophotometer (ThermoSpectronic, Rochester NY, U.S.A.) at 540 nm. A standard curve was made using a 0.2% maltose solution. A unit of α-amylase activity was defined as μg of maltose released per mg of koji solids per minute.

**Protease activities of koji**

The protease activities of koji were determined using the trinitrobenzenesulfonyl acid (TNBS) method as described by Adler-Nissen (1979) with modifications. Five ml of 0.5%
casein (Sigma Aldrich) solution, in 0.21 M sodium phosphate buffer (pH 8.20), was incubated with 1 mL of extracted enzyme sample described above at 37 °C for 30 min with agitation at 80 rpm (Chutmanop and others 2008). Then 2 mL of the hydrolysate was mixed with 18 mL of pre-warmed 1% sodium dodecyl sulfate (SDS), and incubated at 75 °C for 20 min with agitation at 80 rpm. The hydrolysate and SDS mixture (0.25 mL) was added into 2 mL of 0.21 M sodium phosphate buffer (pH 8.20) and 2 ml of 0.1% TNBS (Sigma Aldrich) solution, and the sample was incubated for 1 h at 50 °C with agitation at 80 rpm. Four mL of 0.1 N HCl was then added, and the samples were allowed to cool for 30 min before the absorbance was read using the spectrophotometer described above at 340 nm. The standard curve was made using a 5 mM leucine solution and then converted to μmols of leucine. One unit of enzyme activity was defined as μmols of leucine liberated per g of koji solids in 30 min.

**Total mold counts**

One gram of each type of koji sample was added into 9 ml of 0.1% peptone water and vortexed for 30 sec. The samples were serially diluted in 0.1% peptone water, and the last three dilutions (0.1 mL) were plated in duplicate onto potato dextrose agar (BD Difco, Franklin Lakes, NJ, U.S.A.). The inoculated plates were incubated at 28 °C for 3 to 5 d (DIFCO and BBL, 2011). The colonies on the plates were enumerated and reported as log CFU/g koji solids.

**Statistical Analysis**

Two independent trials were conducted for the koji fermentation and storage studies. For each trial, duplicate samples were included and assayed. Data obtained was analyzed using the Statistical Analysis Software (version 9.1). A two-way analysis of variance (ANOVA), along
with fisher’s least significant difference test (LSD) was used to determine the significance of
difference in product pH, $a_w$, moisture content, and enzyme activity, as well as total mold counts,
based on a confidence level of 95%.

**Results and Discussion**

**Characteristics of rice and barley koji during fermentation**

Table A1 shows the pH, $a_w$, and moisture contents of rice and barley koji during the 118 h fermentation process. The $a_w$ of steamed rice and barley, 0.98 and 0.99, respectively, decreased over time during the fermentation process. At the end of the fermentation, the $a_w$ of rice and barley koji were 0.76 and 0.74, respectively which were significantly different from the $a_w$ of the starting materials (Table A1). The moisture contents of steamed rice and barley were 35.16% and 44.39%, respectively and decreased significantly over time throughout the fermentation process (Table A1). While the pH of fermented barley slurry fluctuated during fermentation, the pH of the rice slurry decreased over time, with the final product pH being 1.23 U lower than the pH of the starting material (Table A1).

Chutmantop and others (2008) found that the moisture contents of rice and wheat bran koji increased during fermentation. The authors attributed this increase to the respiration of the mold which produced carbon dioxide and water during fermentation. Opposite results were however, observed in the present study, and the decrease in the moisture contents of koji may be related to the fermentation equipment used. The fermentation done by Chutmantop and others (2008) took place in glass flasks while this study used wire mesh trays lined with a cloth. Koji in the wire trays had a larger surface area in contact with air of declining relative humidity, which
may have contributed to the gradual decrease in the moisture contents of koji during fermentation. In addition, periodic withdrawal and hand mixing of the product as well as ventilating koji incubation room could also have an impact on the water activity of koji. Lu and others (2003) observed the relationship between moisture content and water activity of wheat bran and soybean meal prepared with *A. sulphureus* and found that water activity was closely related to moisture content and as the moisture content decreased, the water activity also decreased.

Results of the present study showed that *A. oryzae* entered the stationary growth phase in rice koji after 58 h into the fermentation process, which was approximately the time when the α-amylase activity peaked at 116.57 U (Figure A1A). After this sampling point, the α-amylase activity gradually declined as the mold counts remained fairly stable in the stationary growth phase. The barley koji achieved a peak α-amylase activity of 211.87 U after 46 h into the fermentation process, which was also the time when the mold culture entered the stationary growth phase (Figure A1A). Similar to the rice koji, the α-amylase activity of barley koji started to decrease once the mold culture entered the stationary growth phase (Figure A1A). The protease activities of rice and barley koji were low and remained essentially at similar levels during fermentation, although the rice samples taken at 46-70 h had slightly lower protease activities (Figure A1B).

Results of previous studies suggest that the time required for α-amylase to reach a peak activity in koji depended on environmental conditions, starting materials, and strains of *A. oryzae* used in fermentation. Chou and Rwan (1995) found that the α-amylase of *A. oryzae* strain 30428 in rice koji with a moisture content of 50% reached a peak activity of 40.00 U at 60 h into the fermentation. Using a different substrate and strain of *A. oryzae* (Ozykat-1), Chutmanop and
others (2008) observed the maximal α-amylase activity in a rice bran koji within 12 h into the fermentation process. Figure A1A demonstrated that the α- amylase activity and mold counts follow a similar trend and moved in concert with each other until the peak enzyme activity was reached. Similar finding has been reported in a study on spoilage bacteria, and the activities of hydrolytic enzymes peaked as the bacteria entered the stationary growth phase (Braun and Sutherland 2003).

*A. oryzae* has long been used for the production of proteases (Wang and others 2005). Other studies reported high protease activities of koji made with soybeans, wheat bran, wheat flour, or rice bran (Su and others 2005; Nakadai and Nasuno 1988; Chutmanop and others 2008). In this research, however, low protease activities were observed in samples taken throughout the entire fermentation process (Figure A1B). This phenomenon might be related to the low protein contents of the substrates used in the study. Polished, cooked white rice has a protein content of 2.36 g/100 g while cooked barley has a protein value of 2.26 g/100 g (USDA, 2010). In comparison, rice bran, wheat bran, wheat flour, and soybeans have a protein content of 13.35, 15.55, 13.21, and 16.64 g/100 g, respectively (USDA, 2010). The low protein contents in the substrates used in this study may have failed in inducing the expression of proteases in *A. oryzae*. Narahara (1994) found that *A. oryzae* W20 produced relatively small amounts of protease compared with *A. oryzae* M01 on koji made from some of the rice used in the study.

**Characteristics of koji during storage at different temperatures**

Table A2 displays the changes in average pH, a$_w$, and moisture contents of rice and barley koji during storage at -20, 4, or 32 °C. Statistical analyses revealed that the average pH, a$_w$, and moisture contents of rice koji were significantly different from those of barley koji during storage. The differences in average product a$_w$ and pH at -20, 4, or 32 °C were
statistically significant. However, the average moisture contents of koji did not change significantly during storage at the 3 different temperatures. In a previous study, a significant decrease in both pH and moisture content were observed during a six month storage period of wheat grain at 25 and 45°C (Ruska and Timar 2010).

The protease activities in rice vs. barley koji and in koji stored at the 3 different temperatures did not change significantly (Table A2). However, the differences in α-amylase activities in rice and barley koji were statistically significant (Table A2). Under all 3 storage conditions, the barley koji had a significantly higher α-amylase activity compared to the rice koji, which is consistent with what was observed during the fermentation process. A previous study by Sivaramakrishnan and others (2007) found that among fourteen different substrates used in fermentation, some substrates better supported α-amylase production compared to other substrates. Different grains have different sets of nutrients (carbohydrates, proteins, vitamins, minerals), different buffering capacities, and potentially different growth inhibitors (Hammes and others 2005), which all may have an influence on the activity of α-amylase.

The α-amylase activity of rice and barley koji decreased by 49.73 and 20.97 U, respectively during the 9 d storage period at 32 °C (Figure A2A). Within the same time frame, the total mold counts in rice koji decreased by 1.72 log CFU/g. It is likely that once the mold entered the stationary phase, the nutrients had been depleted and the A. oryzae began to die. The decrease in mold metabolism may have a direct impact on the activity of its enzymes. The α-amylase activities and mold counts remained essentially unchanged over the 12 mo storage period at -20°C (Figure A2C). At 4 °C, the α-amylase activity of rice and barley koji increased by 29.12 and 15.89 U, respectively in 10 wk (Figure 2B), but the counts of A. oryzae did not change significantly. The precise reason for the increases is unknown. Since the enzyme
activities and mold counts were calculated based on the dry weights of koji, loss of moisture during fermentation and storage should not have a significant impact on the reported results. These results were in agreement with the findings of Ruska and Timar (2010) who reported that amylase activity decreased as the storage progressed. The decrease of amylase activity was extremely slow at 10°C whereas it was comparatively higher at 45°C than 25°C during six months of storage.

Conclusion

Hydrolytic enzymes are routinely used by food processors to break down starches and proteins in food products. A properly produced and maintained koji with a high hydrolytic enzyme activity can serve as an important source of the enzymes for the food industry. Using the conditions specified in the present study, barley and rice koji fermented for 46 and 58 h, respectively, would provide a product with optimal α-amylase activities. It seems that both starting materials and storage conditions have a significant influence on the a_w, pH, and α-amylase activity of rice and barley koji. However, it is not clear whether the environmental conditions, starting materials, and strain of A. oryzae used in the present study were among the contributing factors for the low protease activities observed in the present study. Further studies are needed to investigate the possible influence of these factors on protease activities.
Acknowledgment

The authors would also like to thank Glenn Ferrell and Jerry Davis for assistance. The project was made possible through a research grant provided by the USAID.

References


Difco and BBL Manual [Internet]. 2nd ed. Sparks, MD: Becton, Dickinson and Company

[Accessed 2011 Dec 3]. Available from:


Table A1 - Physical characteristics of rice and barley koji during fermentation process.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Water Activity rice</th>
<th>Water Activity barley</th>
<th>Moisture Content (%) Rice</th>
<th>Moisture Content (%) Barley</th>
<th>pH Rice</th>
<th>pH Barley</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.98a</td>
<td>0.99a</td>
<td>35.16a</td>
<td>44.39a</td>
<td>6.37a</td>
<td>5.29h</td>
</tr>
<tr>
<td>10</td>
<td>0.97a</td>
<td>0.98ab</td>
<td>34.07a</td>
<td>43.41a</td>
<td>5.99b</td>
<td>5.33gh</td>
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<tr>
<td>22</td>
<td>0.95ab</td>
<td>0.97ab</td>
<td>33.57a</td>
<td>42.22b</td>
<td>5.20ef</td>
<td>5.38g</td>
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<tr>
<td>34</td>
<td>0.93bc</td>
<td>0.96bc</td>
<td>30.26b</td>
<td>36.46c</td>
<td>5.55d</td>
<td>6.15e</td>
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<td>30.17d</td>
<td>5.86bc</td>
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<td>58</td>
<td>0.88d</td>
<td>0.93c</td>
<td>23.50d</td>
<td>25.20e</td>
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<td>21.91f</td>
<td>5.49d</td>
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<tr>
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<td>0.87de</td>
<td>20.09ef</td>
<td>19.32g</td>
<td>5.34e</td>
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<td>17.00h</td>
<td>5.27ef</td>
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<tr>
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<td>0.82f</td>
<td>17.34g</td>
<td>15.67i</td>
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<td>0.74g</td>
<td>13.81h</td>
<td>13.77j</td>
<td>5.14f</td>
<td>5.98f</td>
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Means (n = 4) followed by different letters in the same column are significantly different ($P < 0.05$).
Table A2 – Average water activities, moisture contents, pH, and enzyme activities of koji during storage at 32, 4, and -20 °C.

<table>
<thead>
<tr>
<th>Type of koji</th>
<th>Water activity</th>
<th>Moisture Content (%)</th>
<th>pH</th>
<th>α-amylase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protease activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rice</td>
<td>0.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Storage temperature (°C)

| -20 | 0.91<sup>a</sup> | 26.58<sup>a</sup> | 5.73<sup>a</sup> | 97.04<sup>b</sup> | 0.01<sup>a</sup> |
| 4   | 0.90<sup>b</sup> | 26.48<sup>a</sup> | 5.69<sup>b</sup> | 102.65<sup>a</sup> | 0.01<sup>a</sup> |
| 32  | 0.88<sup>c</sup> | 26.56<sup>a</sup> | 5.05<sup>c</sup> | 59.55<sup>c</sup> | 0.01<sup>a</sup> |

Means followed by different letters within each parameter in the same column are significantly different (n = 12; P < 0.05).

<sup>a</sup> One unit of α-amylase activity was defined as μg of maltose released from per mg of koji solids per minute

<sup>b</sup> One unit of protease activity was defined as μmols of leucine liberated from per g of koji solids in 30 min.
Figure legends

Figure A1 - \( \alpha \)-amylase (A) and protease (B) activities of rice (-■-) and barley (-●-) koji in relation to the total mold counts (rice -□- and barley –○-) during the fermentation process. The activity of \( \alpha \)-amylase for both types of koji is defined as \( \mu \)g maltose liberated per mg of koji solids per min compared with the total mold counts (log CFU/g). Protease activity is defined as \( \mu \)moles of leucine liberated per g of koji solids in 30 min compared with the total mold counts (log CFU/g).

Figure A2 - \( \alpha \)-amylase activity of rice (-■-) and barley (-●-) koji compared with total mold counts (rice -□- and barley –○-) during storage at 32 °C (A), 4 °C (B) and -20 °C (C). The activity of \( \alpha \)-amylase for both types of koji is defined as \( \mu \)g maltose liberated per mg of koji solids per min compared with the total mold counts (log CFU/g).
Fig A1. Bechman et al.

A

B
Fig. A2 Bechman et al.

A

![Graph showing α-amylase activity and Log (CFU/g koji solids) over 9 days.]

B

![Graph showing α-amylase activity and Log (CFU/g koji solids) over 12 weeks.]

C

![Graph showing α-amylase activity and Log (CFU/g koji solids) over 12 months.]

Time (Days)

Time (Weeks)

Time (Months)
APPENDIX B

Descriptive Sensory Ballot
Sample_____           Panelist_____

Please rinse mouth with water and cracker between each sample. Taste the product sample (1-2 spoonfuls at a time for the flavor descriptors and 1-2 spoonfuls at a time for the textural descriptors) and mark a vertical line on the horizontal line below that corresponds with the intensity of each descriptor. The line that is marked indicates the standard given wherever applicable. **DO THE OXIDIZED SAMPLE FIRST BY OPENING THE CUP SLIGHTLY AND SMELLING THE SAMPLE.**

### Sweetness: (Sucrose standard solutions = 20, 50, and 100)

<table>
<thead>
<tr>
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<th>20</th>
<th>50</th>
<th>100</th>
<th>138</th>
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<tbody>
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<tr>
<td>Extreme</td>
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### Bitterness: (Caffeine standard solutions = 20, 50, and 100)

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<th>100</th>
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<td>Extreme</td>
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### Peanut Flavor: (Medium roasted peanuts = 70)

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<tbody>
<tr>
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<tr>
<td>Intense</td>
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### Pea Flavor: (Canned Blackeye Peas = 60; Canned Chick Peas=100)

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<tr>
<td>Intense</td>
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### Oxidized: (Microwaved vegetable oil=80)

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<tr>
<td>Extreme</td>
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</table>

### Thickness: (50% Karo syrup solution = 50; Karo Syrup = 120)

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<tr>
<td>Thick</td>
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</table>

### Graininess/Grittiness: (NJ Red= 30; NJ Mango Veggie=55; NJ Protein Zone=110)

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<tr>
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### Other Off Flavors:
Please write down any other off flavors detected in the product.