Cape Peninsula University of Technology

Evaluation of bambara groundnuts (*Vigna subterrenea* (L.) Verdc.) milk fermented with lactic acid bacteria as a probiotic beverage

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Thesis presented in partial fulfilment of the requirements for the degree of

Master of Technology (Food Technology)

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September 2012

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entire or part, been submitted at any other university for a degree.

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ABSTRACT

The aim of this study was to evaluate bambara groundnut milk (BGNM) subjected to fermentation with lactic acid bacteria (LAB) as a probiotic beverage with a view to developing value-added product. Central Composite Rotatable Design (CCRD) was used to optimise the hydration time and temperature of BGN flour for optimum BGN milk (BGNM) production. The optimum time and temperature was 2 h at 25^{o}C. The effect of variety was assessed on the quality and consumer acceptability of BGNM prepared from five varieties of BGN (black, red, brown, brown-eye, and black-eye) which were representatives of the BGN available in South Africa. BGNM from the five varieties differed significantly ($p<0.05$) in, lightness, chroma, redness, yellowness, hue and antioxidative activity, while the pH were not significantly different. The four BGNM samples were significantly different ($p < 0.05$) in appearance, colour, mouthfeel and overall acceptability but not in aroma and taste. A three factor design (4 x 3 x 3) consisting of probiotics (*Lactobacillus acidophilus, L. bulgaricus, L. casei* and *L. plantarum*), temperature and fermentation time, were used to estimate the optimal conditions for the production of BGN probiotic beverage (BGNPB). The optimal condition for the production of BGNPB was estimated to be 35^{o}C for 24 h with a desirability of 0.854 for *L. bulgaricus*. The next promising probiotic was *L. plantarum* that could be fermented at 35^{o}C for 24 h with 0.843 desirability. BGNM from the red variety were fermented with *L. bulgaricus* and *L. plantarum and L bulgaricus (*in combination*),* making plain and sweetened BGNPB which were evaluated for their quality and consumer acceptability. The four BGNPB samples were significantly different ($p < 0.05$) in aroma, taste, mouthfeel and overall acceptability but not in appearance and colour. The plain BGNPB were assessed for their proximate composition, antioxidant activity, *in vitro* probiotic tolerance to simulated gastric juices and bile and a 28 days shelf life study at 5, 15 and 25^{o}C. The protein, total dietary fibre (TDF), ash and antioxidative activity of the BGNPB were significantly different while the fat and carbohydrates were not significantly different. Time and concentration of the gastric juice and bile had significant effects on the percentage bacterial survival of probiotics in the BGNPB. However, the probiotics did survive, in low numbers, in the simulated gastric juice and bile after 180 and 240 minutes of incubation. Titratable acidity, pH, microbial load and colour of the BGNPB were

significantly affected by the storage time and temperature during the shelf life study. At the 5^{o}C storage temperature the BGNPB had a right censored shelf life on day 28. At 15^{o}C the shelf life was 18 and 10 days for *L bulgaricus* and *L. plantarum* and *L. bulgaricus* respectively. The outcome of this research showed that a novel BGNPB product can be made from fermenting BGNM with LAB.

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Language and style used in this thesis are in accordance with the requirements of the International Journal of Food Science and Technology. This thesis represents a compilation of manuscript where each chapter is an individual entity and some repetition between chapters have, therefore, been unavoidable.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and institutions that formed an integral part of the completion of this research study:

Prof. V.A. Jideani, my supervisor and lecturer at the Cape Peninsula University of Technology, for her guidance, patience, support and assistance during the course of my studies;

Cape Peninsula University of Technology University Research Funding (URF) afor the financial assistance;

Mrs. L. Cloete, Microbiology laboratory technician at the Cape Peninsula University of Technology Bellville campus, for her help with the procurement of chemicals and consumables.

The financial assistance of the National Research Foundation towards this research is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation and the University Research Funding.

DEDICATION

Dedicated to the Lord God my Heavenly Father who made me for His own glory, and my family, my mother Balbina, late father, Noah, my siblings Nyaradzo, Gerald and Belinda and aunt, Evelyn with bottomless gratitude for their love, support and believing in me, last but not least Knowledge Zengeya for being there.

CHAPTER 1
MOTIVATION AND DESIGN OF THE STUDY

1.1 INTRODUCTION

Introduction of exotic crops has led to the neglect of many indigenous African crops (Odeigah & Osanyinpeju, 1998). Agricultural research traditionally has focused on staple foods while little attention has been given to underutilised and neglected crops, particularly by scientist in developed countries (Heller *et al*., 1997). Nevertheless, empirical evidence and the result of specific research programmes indicate that these underutilised crops have considerable potential (Lawal *et al.,* 2007) in food security, but more scientific studies needs to be channelled to these crops (Odeigah & Osanyinpeju, 1998). One such underutilised crop is bambara groundnut (BGN). BGN is known in South Africa as jugoboon in Afrikaans; jugo in Xhosa; indlubu in Ndebele; nduhu in Venda; ditloo in Sesuthu; nyimo in Shona (Zimbabwe) and okpa otuanya in Nigeria. BGN is an indigenous African crop that has been cultivated in Africa for years. It is a highly nutritious plant that plays a crucial role in people's diets (Jideani & Mpotokwane, 2009). It is grown in the African continent from Senegal to Kenya, and from the Sahara to South Africa and Madagascar (Swanevelder, 1998). Beyond Africa, BGN is cultivated in Brazil where it is known as 'mandubi d'Angola' as well as in West Java and southern Thailand. Other tropical locations such as Middle East, Syria and Greece could also grow BGN. Small-scale cultivation trials of BGN have been successful in Florida, United States (NRC, 1996).

BGN has become less important in many parts of Africa because of the expansion of American groundnut (*Arachis hypgaea*) production (Heller *et al.,* 1997; Odeigah & Osanyinpeju, 1998). In recent years there has been renewed interest in the crop for cultivation in the arid savannah zones, because of its resistance to drought, pests and the ability to produce a reasonable crop when grown on poor soils and has long storage life (NRC, 1996; Swanevelder, 1998; Massawe *et al.,* 2002). Whole bambara groundnuts (BGNs) are edible seed of the BGN plant, a legume from the *Leguminosae* family (Heller *et al.,* 1997; Basu *et al.,* 2007). They are low in fat, but a good source of protein (Jideani & Mpotokwane, 2009) and believed to rank the third grain legume after groundnuts and cowpea

(*Vigna unguiculata)* (Amarteifio & Moholo, 1998; Adebowale *et al.,* 2002). The seed contains about 49-63.5% carbohydrate, 15-25% protein, 4.5-7.4% fat, 5.2-6.4% fibre, and 3.2-4.4% ash (Goli *et al.*, 1997; Karikari *et al.,* 1997; Amarteifio & Moholo, 1998). BGN is a good source of fibre, calcium, iron, potassium and is unusually high in methionine, an essential sulphur-containing amino acid (Omoikhoje, 2008).

Protein foods particularly animal protein continues to be in short supply in the diets consumed by a large segment of the population in developing countries. Legumes are some of the low priced source of protein rich food important in alleviating protein malnutrition and shortages (Apata & Ologhobo, 1994).

The health trend by consumers, together with the drive to exploit the full potential of home grown legume seed crops, has provoked the utilisation of relatively neglected legume sources for human consumption and as livestock feed components (Apata & Ologhobo, 1994). Vegetable milk has been proved to be a prospective alternative to animal milk in the food industry, due to their functionality and health claims (Uvere *et al.,* 1999; Lawal *et al.,* 2007; Nti, 2009). The possibility of producing vegetable milk for local use was studied by Brough *et al.* (1993) and the results indicated a great potential for the BGNM. Vegetable milk from legumes and nuts can be extracted giving products which can surrogate animal milk (Nsofor *et al.,* 1996). Milk extraction can be done on soaked seeds which are wet milled and then strained (Nsofor *et al.,* 1996). It is known that beany flavour is associated with milk extracted from legumes and nuts making them unpopular (Brough *et al.,* 1993). Nevertheless, fermentation; malting and heat treatment can effectively eliminate the beany flavour (Uvere *et al*., 1999; Isanga & Zhang, 2009; Nti, 2009). Many studies have reported that soybean (*Glycine max*) products fermented with lactic acid bacteria (LAB) can provide unique probiotic foods for human nutrition (Kneifel *et al*., 1993; Heller, 2001; Oliveria *et al*., 2001; Shimakawa *et al.,* 2003; Granato *et al.,* 2010). Like soybean products, lactic acid fermented BGN may be suitable probiotic bacterial strain carrier to the host. Numerous studies have been done on BGNs (Igbedioh *et al.,* 1994; Alobo, 1999; Amadou *et al.,* 2001; Adebowale *et al.,* 2002; Jideani & Motokwane, 2009; Alakali *et al.,* 2010) however, nothing is known about bambara groundnut milk (BGNM) as a probiotic beverage. The objective of this study was

to evaluate BGNM fermented with LAB as a probiotic beverage with a view to developing value-added product.

1.2 Statement of research problem

Inspite of the growing importance of BGN, much of the use of bambara in South Africa is limited to boiling and roasting of the seeds. To tap into bambara as a good source of fibre, calcium, iron and potassium and methionine, there is a need for value-added products from BGN. Vegetable milk types have been proved to be a prospective alternative to animal milk due to functionality and health benefits. Therefore it is of interest to assess the potential BGNM as a probiotic beverage.

1.3 Broad objective

The aim of this study was to evaluate BGNM fermented with LAB as a probiotic beverage with a view to developing value-added product.

1.3.1 Specific objectives of the research

Specific objectives of the research include

1. Optimisation of the BGNM production.
2. Production of consumer acceptable BGNM.
3. Optimizing the survival of LAB in BGNM and identifying the best strain for fermentation.
4. Developing a BGN probiotic beverage (BGNPB) which will have viable cell count not less than 10^7 cfu per mL at the end of shelf life to conform to the CODEX Standards for fermented milk (Anon, 2003).
5. Assess the proximate composition and the antioxidative activity of the BGNPB.
6. *In vitro* evaluation of BGNPB for LAB survival in simulated gastric juice and bile.

1.4 Research hypotheses

1. Based on the work of Brough *et al*. (1993) BGNM will be acceptable to the consumer.
2. Different LAB strains will show different growth and survival patterns in BGNM (Wang *et al.,* 2009).

3. The BGNPB will have viable cell count not less than 10^7 cfu per mL at the end of shelf life
4. LAB in the BGNPB will survive *in vitro* simulated gastric juice and tolerate the bile (Gotteland *et al*., 2006; Shobharani & Agrawal, 2009).

1.5 Delineation of the research

1. Four strains of *Lactobacillus* bacteria were used, namely, *Lactobacillus acidophilus; Lactobacillus bulgaricus; Lactobacillus casei* and *Lactobacillus plantarum.*
2. Five landraces of BGN (black, brown, red, brown-eye and black-eye) were used in the study.

1.6 Significance of the research

Novel forms of BGN utilization might encourage its wide production, hence improving its economic status. According to Isanga & Zhang (2009), preparation and fermentation of vegetable milk may serve as one such effort that can increase the consumption of this viable crop and hence improve its commercial status. Furthermore, potential value-added product such as the BGNPB has the potential to cut the heart of Africa's great problems in rural development, hunger, malnutrition and gender inequality.

BGN is a low cost crop, and grown by families for their own subsistence and for their annual income. BGN probiotic beverage will result in buoyant new market outlets for farmers and the boosting of income opportunities for rural areas (NRC, 1996). BGN is a resilient and reliable crop that thrive very well in sites too hot and too dry where peanut, maize, or even sorghum will not grow well. It is also described as a complete food owing to its exceptional nutritional quality (NRC, 1996; Mahala & Mohamed, 2010).

Compared with peanut, BGN has less oil and slightly less protein, but more carbohydrate, and antioxidative potential (Pale *et al,* 1997). The overall combination nicely balances the food groups. People can live on BGN, an uncertain proposition for other legumes (NRC, 1996). Hence, it could be a tool for attacking Africa's chronic malnutrition.

BGN has been mostly grown by women. It therefore offers a convenient way for economically empowering women, thereby improving the lives of their families.

Similarly, BGN offers good opportunities for gender-oriented innovation and commercial development (NRC, 1996).

1.7 Expected outcomes, results and contributions of the research

Novel knowledge on the possibility of producing vegetable milk from BGN through hydration of BGN flour as rather than wet milling will be generated. Information regarding the survival of probiotics in BGNM, the best strain for production of BGNPB and the fermentation of the milk with LAB to produce BGNPB will also be generated. The potential of BGN as a vehicle for providing probiotics to humans will be established.

At least one article will be sent for publication, and the research output will be presented at least at one international conference.

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CHAPTER 2
LITERATURE REVIEW

2.1 Origin and description of BGN

Bambara groundnut (BGN) belongs to the *Leguminosae*, subfamily *Papilionodease* (Heller *et al.,* 1997; Basu *et al.,* 2007). Botanical studies showed great similarities between BGN and plant species of the genus *Vigna*. This confirmed the current name *Vigna subterranea* (L.) Verdc proposed by Verdcourt (1980). According to Swanevelder (1998) there are two botanical varieties namely *V. subterranea* var *spontanea* which includes the wild varieties and *V. subterranea* var s*ubterranea* which includes the cultivated varieties. Investigators interested in the origin of the BGN (Heller *et al.,* 1997; Amadou *et al.,* 2001; Massawe *et al*., 2002; Basu *et al.,* 2007) all agreed that the crop originated from the African continent. The common name actually appears to be derived from a tribe, Bambara, who now live mainly in Mali (Goli *et al.*, 1997). BGN resembles the American groundnut (Odeigah & Osanyinpeju, 1998) in both cultivation and habit, is related to cowpeas (Swanevelder, 1998), and has chemical similarities with soybean (Amaefulu & Osuagwa, 2005). It is one of the five important protein sources for many Africans (Basu *et al.,* 2007).

BGN is a herbaceous, intermediate, annual legume that self-pollinates (Heller *et al*.,1997; Basu *et al.,* 2007) with a compact well developed tap root with many short (up to 20 cm long) lateral stems on which leaves are borne as shown in Figure 2.1. Leaves are trifoliate, petiole is long, stiff and grooved and the base is green or purple in colour (Basu *et al*., 2007). Flowers are typically *papilionaceous* and are borne in a raceme on long, hairy peduncles which arise from the nodes on the stem (Massawe *et al*., 2002). The plant has well developed tap root with profuse geotropic lateral roots. Its podding habit is similar to that of groundnut in that pale yellow flower stalk bends downwards after fertilisation, pushing the young developing pod into the soil, where it will develop and mature (Amadou *et al*., 2001) or just above the ground (Basu *et al*., 2007). The pods are spherical or oval in shape and may reach up to 3.7 cm (Figure 2.1) depending on the number of seeds they contain (Basu *et al.,* 2007). Most cultivars have single seeded pods, but pods with three seeds are frequently found in the ecotype from

(a)

(b)

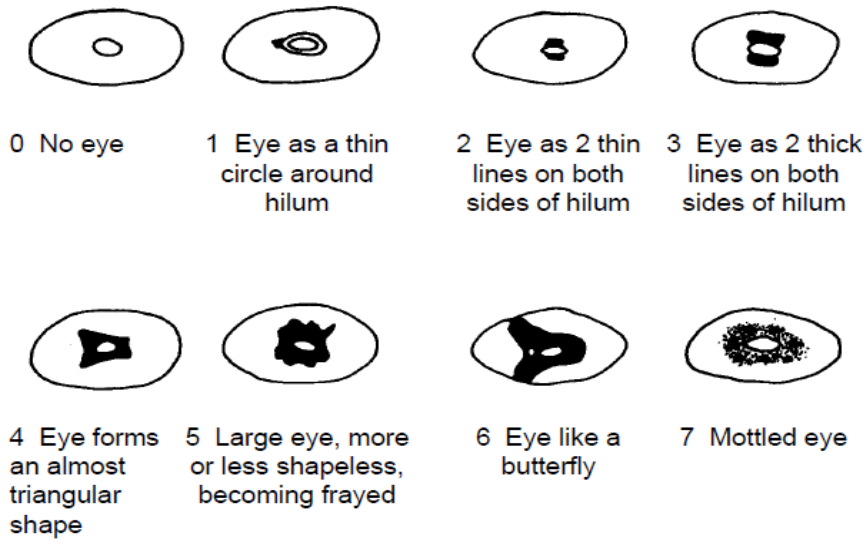
Figure 2.1 Bambara groundnut (a) plant (b) pods (Anon 2011).

Congo (Amadou *et al.,* 2001). The unripe pod is yellowish green while the mature pods are indehiscent, often wrinkled, ranging from yellowish to a reddish dark brown colour (Basu *et al.,* 2007). The varieties consist of several genotypes which have different capacity to tolerate biotic and abiotic stress under a low input agricultural system (Zeven, 1998; Amadou *et al.,* 2001; Basu *et al.,* 2007). An average day temperature of 20-28^{o}C is ideal for the crop; it has a growth period of 110 to 150 days for the crop to develop (Basu *et al.,* 2007). BGN will grow on any well drained soil, but light sandy, loams with a pH of 5.0-6.5 are most suitable (Basu *et al.,* 2007). Amadou *et al*. (2001) stated that BGN will often yield well in environments that may be too hostile for more favoured legumes, it is not prone to the risk of total crop failure, especially in low and uncertain rainfall (Brough *et al*., 1993; Baryeh, 2001), making it play an important socio-economic role in the semi arid regions of Africa (Massawe *et al.,* 2002). In this era of global warming and food security threats in Africa, BGN will be a crop of hope to alleviate malnutrition and poverty as a result of its drought tolerant characteristics.

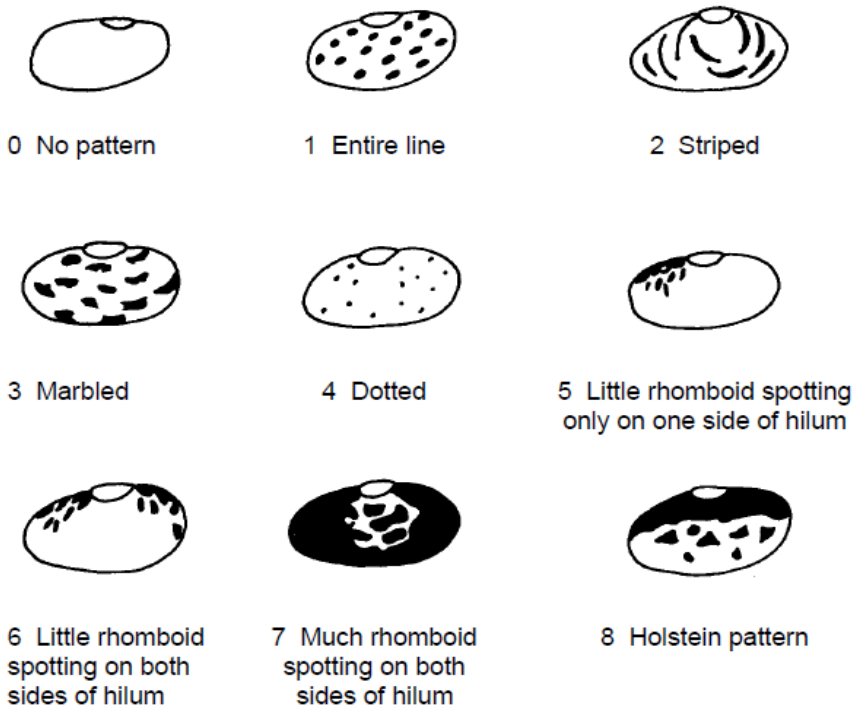
2.2 Physical properties of BGN seeds

Physical properties of BGN affect how they are to be processed, handled, stored, and consumed (Baryeh, 2001; Mpotokwane *et al.,* 2008). The physical properties are also required for the design, evaluation of equipment and systems for their handling (Mpotokwane *et al*., 2008; Jideani & Mpotokwane, 2009), and they give an indication of its processing characteristics leading to possible value added products (Baryeh, 2001; Mpotokwane *et al.,* 2008).

BGN varieties have recognizable morphological features like the testa colour and patterns which can be used for their identification (Massawe *et al.,* 2002). Various testa patterns are found, including mottled, with or without hilum (eye) coloration, blotched or stripped, with or without hilum coloration. Figure 2.2 shows some of the eye and testa patterns of BGN varieties (Goli *et al.,* 1997). Figure 2.3 shows the different varieties, A to E used in this study. Commonly, varieties have names based on the testa and the place they are grown (Massawe *et al.,* 2002). Five varieties of BGN with distinct colour difference namely, cream black eye; cream white eye; brown; maroon and black were studied by Nti (2009). The two cream varieties have smaller seed size, in terms of thousand seed weight, and they are reported to have thinner seed coats (0.11 and



(a)



(b)

Figure 2.2 Types of bambara groundnut (a) eye patterns and (b) testa patterns (Goli *et al*., 1997).

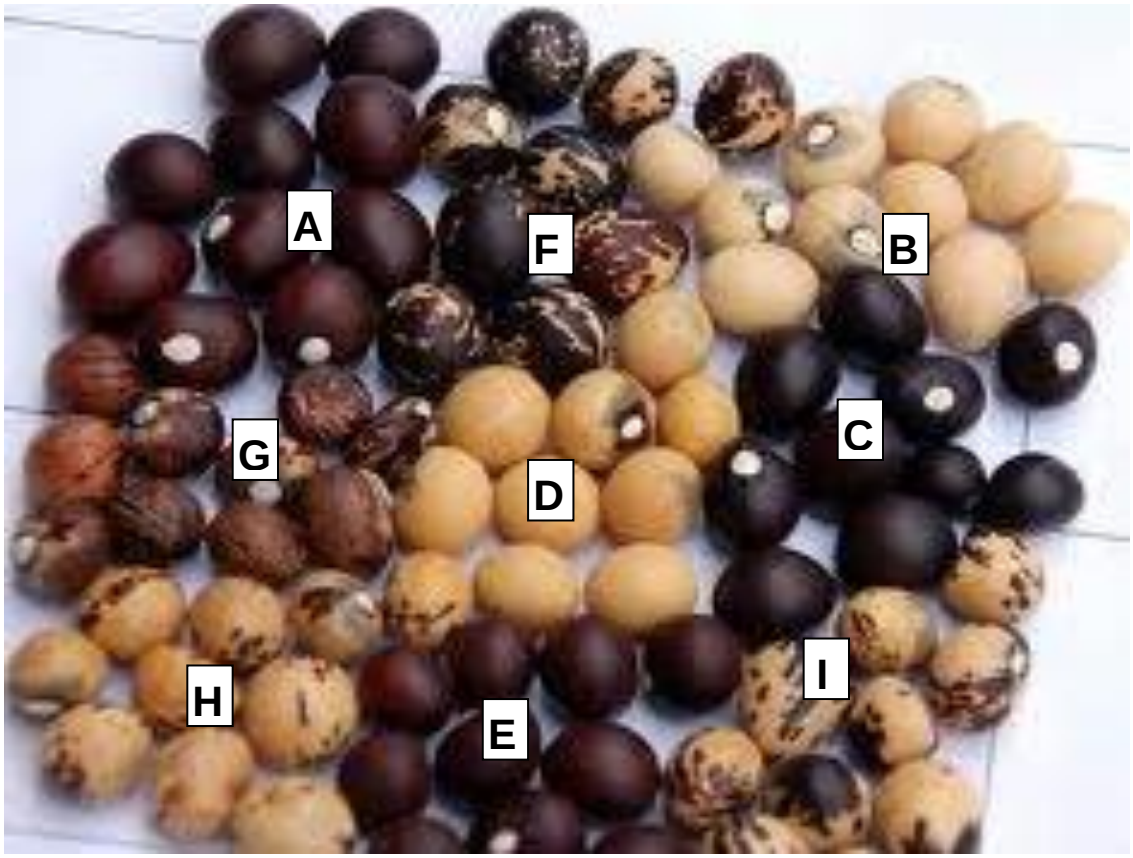


Figure 2.3 Varieties of bambara groundnuts, A - brown; B - brown eye; C - black; D - black eye and E - red which were used in this work, while F - I named according to testa pattern (Anon 2010a).

0.12 mm), than the other three dark colour seeds with thicker coat (0.20 mm). The germ length constitutes 55-72% of the total seed length while germ width was 50-70%. Mpotokwane *et al.* (2008) reported on the physical properties of five BGN [National Tested Seed Red (NTSR); Botswana Red (BoTR); Diphiri cream (DipC1 and DipC2) and speckled (AS17)] from Botswana. NTSR are longer while Dip2 (brown eye) are shorter in length than others. BGN seeds differed significantly in geometric mean diameters, volume and surface area with NSTR, BoTR and DipC1 (black-eye) being higher.

The seeds are generally high in aspect ratio, being highly spherical with tendency to roll rather than slide. DipC1 (black-eye) seeds are more spherical and occupies more volume and area than others. The speckled seeds (AS17) are heavier than the others followed by DipC1 and DipC2. Generally, the seeds are heavier than water and therefore would not float in water. Jideani and Mpotokwane (2009) reported hydration kinetics of these five BGN seed varieties. The water hydration rate increased and water absorption capacity decreased with increase in temperature. Hydration changes were associated with exothermic and energetically favourable transformation. Higher activation energy and faster water absorption were reported for the speckled varieties (AS17). The optimum soaking time for BGN seeds was reported as 6 h at temperature $\leq 75^{o}$C for all the varieties.

2.3 Economic and food uses of BGN in Africa

BGN is essentially grown for human consumption. The seed makes a nutritious and complete food owing to its chemical composition (Brough *et al.,* 1993; Ijarotimi & Esho, 2009; Mahala & Mohammed, 2010). Several workers have examined the biochemical composition of the seed (Alobo, 1999; Adebowale *et al.,* 2002; Ijarotimi & Esho, 2009; Lawal *et al.,* 2007; Nti, 2009; Mahala & Mohammed, 2010). Despite the low fat content some tribes in Congo roast and pound the seeds to extract oil (Goli *et al*., 1997). BGN are consumed in many ways, eaten fresh, or grilled while immature (Mpotokwane *et al.,* 2008, Goli *et al.,* 1997; Swanevelder, 1998; Uvere *et al.,* 1999).

BGN harden at maturity and therefore require soaking and or boiling before any specific preparation (Abu-Ghannam & McKenna, 1997). The fresh pods are boiled with salt and pepper, and eaten as a snack in many West African countries (Goli *et al.,* 1997). In East Africa, the groundnuts are roasted, then crushed, and

used to make soup, with or without condiments (Uvere *et al.,* 1999 & Goli *et al*., 1997). In Cote d`voire, the seeds are used to make flour, therefore making it more digestible. In Zambia bread is made from BGN flour (Linnemann, 1990); cakes in Botswana (Goli *et al.,* 1997; Lawal *et al.,* 2007); stiff porridge, which has a relatively long shelf-life (Goli *et al.,* 1997). Paste from BGN flour is used in the preparation of steamed product such as o*kpa* in Nigeria (Goli *et al.,* 1997). O*kpa* is cooked dough-like gel made from BGN paste that is wrapped in banana leaves and boiled. Roasted seeds can be boiled, crushed and eaten as relish. In Botswana consumers often prefer the immature seeds which are boiled in pod, salted, and consumed either on their own or with maize seeds. The seeds of mature black variety are used for medicinal purposes (Heller *et al.,* 1997). Table 2.1 is a summary of the culinary uses of BGN in Africa as reported by Goli *et al.* (1997).

Brough *et al.* (1993) studied the potential of making vegetable milk and compared the flavour and composition of BGNM with milk prepared from cowpea, pigeon pea and soybean. BGNM was ranked first, and the lighter colour of the milk was most preferred, giving an indication of the potential of making BGNM.

2.4 Nutritional versatility and desirable functional characteristics of BGN

BGN seed makes a complete food (Brough *et al.,*1993; Ijarotimi & Esho, 2009; Mahala & Mohammed, 2010) as it contains sufficient quantities of protein (20.5%), carbohydrate (63%) and fat (6.3%) with relatively high proportions of lysine (6.6%) and methionine (1.3%) (Brough *et al.,* 1993); appreciable amount of micronutrients (Ijarotimi & Esho, 2009) and has antioxidative properties (Pale *et al.,* 1997; Onyilagha *et al.,* 2009).

Protein and carbohydrates composition of BGN differs with varieties (Basu *et al.*, 2007; Amarteifio & Moholo, 1998; Baryeh, 2001). Protein ranges between 19.3-27.2% (Heller *et al.,* 1997). The black variant has the highest protein content, while the cream has the lowest (Nti, 2009). The high protein content of BGN is an indication of its prospective use as functional ingredients. Alakali *et al.* (2010) evaluated composite beef-BGN flour patties, and reported that the BGN flour significantly reduced the shrinkage of the cooked patties, while percentage cooking yield, moisture retention and fat retention increased significantly. Brough *et al.* (1993) reported that a crude protein isolate (76%) from BGN protein was

Table 2.1 Culinary uses of bambara groundnut in Africa (Goli *et al.,* 1997)

Country	Name	Culinary uses
South Africa	Sekome (Sesuthu) Tihove (Shangaan) Tshidzimba (Venda)	Adding beans and peanuts or just one of the two, to maize or millet meal and boiling the mixture until it forms a stiff dough. This is salted and pounded into a small ball, and will often keep fresh for several days.
	Tshipupu (Venda)	Boiled and then stirred to make a thin porridge, like maize, they may also be added to a porridge made from peanuts.
	Dithaku (Sesutho)	Cooked with maize and pounded into thick sticky dough.
Zimbabwe	Mutakura (Shona) Nkobe (Ndebele)	Boiled with maize, peanuts, cowpeas salted served with *mahewu.*
West African countries		The fresh pods are boiled with salt and pepper, and eaten as a snack
Botswana		Immature seeds, which are boiled in pod, salted, and consumed either on their own or with maize seeds
East Africa		The groundnuts are roasted, then pulverised, and used to make soup, with or without condiments

inferior to the standard protein (egg albumen and soy isolate) in terms of functionality. Nevertheless, the same study reported that BGN milk was most acceptable to the consumers as compared to cowpea, pigeon pea and soybean milk. Nonetheless, Lawal *et al.* (2007) argued that native proteins possess limited functionality and recommended a technical need to manipulate the plant proteins and give them desirable characteristics. The authors improved the solubility of BGN protein concentrate following acetylation and succinylation resulting in improved emulsifying and foaming properties of BGN native protein concentrate. Table 2.2 shows the range of chemical composition of BGN (Apata & Ologhobo, 1994; Igbedioh *et al*., 1994; Ijarotimi & Esho, 2009; Mahala & Mohammed, 2010). Omoikhoje (2008) observed that the essential amino acids content of BGN such as lysine 6.82 g/16 gN; methionine 1.85 g/16 gN and cysteine 1.24 g/16 gN is comparable to that of soy bean lysine 6.24 g/16 gN; methionine 1.14 g/16 gN and cysteine 1.80 g/16 gN. Furthermore, Onimawo *et al.* (1998) reported that BGN has potential as a source of plant proteins due to high (17.5-21.1%) crude protein content. In addition, emulsifying activity, bulk density and oil binding capacities of BGN indicate that it can be used in food formulations for specific functional properties. Hence, native protein functionality might be lacking (Lawal et al., 2007) but still BGN is considered an important crop, and complete food owing to it nutritional composition (Brough *et al.,* 1993; Apata & Ologhobo, 1994; Ijarotimi & Esho, 2009; Mahala & Mohammed, 2010).

Dark seeded varieties (black and red) have higher nutrient and mineral contents than the light-seeded ones (cream) (Goli *et al.,* 1997; Nti, 2009). However, in Botswana the locals prefer the light colour seeds for consumption because they have short cooking times and superior flavour (Heller *et al.,* 1997; Nti, 2009). Mahala & Mohammed (2010) reported crude protein in the seeds (25%) higher than in the pods (19.6%) and hulls. In contrast, Omoikhoje (2008) and Brough *et al.* (1993) reported lower content in the seeds, 19.6% and 16.3% respectively. The disparity may be as a result of differences in soil content as well as the moisture content of the BGN and varieties (Nti, 2009).

Total carbohydrate content of legumes is typically 60% and these carbohydrates generally have good bioavailability (Lam & De Lumen, 2003). Starch account for the most portion of legume carbohydrate content, ranging from 24-56% while the oilseeds have lower carbohydrate content with values such as

Table 2.2 The composition of bambara groundnut (Apata & Ologhobo, 1994; Igbedioh *et al.,* 1994; Ijarotimi & Esho 2009; Mahala & Mohammed, 2010)

Component	Value
Proximate (g/100 g)	
Ash	2.0 - 3.6
Carbohydrate	63.5 - 68.5
Crude fat	1.6 - 6.7
Crude fibre	1.8 - 12.9
Crude protein	17.0 - 24
Minerals (mg/100 g)	
Iron	5.9 - 7.1
Potassium	1240 - 1290
Phosphorus	296 - 320
Sodium	3.7 - 4.8
Calcium	7.8 - 13.5

14% for peanuts and 32% for soybean. Oligosaccharides of the raffinose family (raffinose, starchyose and verbascose) account for 31-76% of the total sugar. The resistance of raffinose oligosaccharides in human and monogastric animal digestion present a flatulence problem to certain individuals (Lam & De Lumen, 2003). Soybean milk naturally does not contain a lot of simple sugars, thus sucrose or glucose can be added during yoghurt production to facilitate the fermentation process. Germination of soy beans to be used in yoghurt production reduces flatulence causing oligosaccharides in soy milk. Hence, germination of BGN seeds can be adopted for producing flatulence-free milk from BGN. BGN is reported to have the highest concentration of soluble fibre among other beans. Soluble fibre is a non-nutrient common in oat bran and is believed to reduce the incidence of heart disease and to help prevent colon cancer (NRC, 1996).

Fat content of BGN seeds (6 - 8% of dry matter) is higher than that found in cereals; it is too low to be used as an oil source (Brough *et al.,* 1993). However, a tribe in Congo roast and pound the seeds to extract oil (Goli *et al.,* 1997). Fat is higher in the hull and pods while least in the seeds, 4.3%, 3.5%, and 1.6% respectively (Mahala & Mohammed, 2010). The lipid content of legumes is generally 7% with the exception of the oil seeds, peanut and soybean, which contain about 52 and 20% oil respectively. Legumes provide essential fatty acids to the human diet. Peanut contains 22% linolenic acid, whereas soybean has 54% linoleic acid and 8% linolenic acid (Aidoo *et al.,* 2010). BGN has high lipid content relative to cowpeas and hence constitutes a potential source of essential fatty acids, especially linoleic (Alain *et al.,* 2007).

The ash content of BGN is reported to be higher in the hulls (5.3%) than in the seeds (3.6%) (Mahala & Mohammed, 2010). Black variant has the highest iron of 6.6 mg/100 g, while 3.3-3.9 mg/100 g was recorded for the cream coloured variant. Legumes are high in iron, with one serving of legume providing around 2 mg of iron. This compares favourably with the iron Recommended Daily Allowances (RDAs) of 10 and 15 mg for adult men and pre-menopausal women respectively. However, iron bioavailability from legumes is poor; as a result their value as a source of iron is diminished (Lam & De Lumen 2003). In contrast, zinc bioavailability from legumes is relatively high at around 25%. Furthermore, many beans are good sources of calcium, providing on the average 50 mg of calcium per serving (Lam & De Lumen 2003). Calcium bioavailability from soybean

products is essentially equivalent to that from milk, but the balance of calcium to phosphorus is inadequate, since the ratio is 1:2, whereas the ideal is 2:1. Legume contain very low (0.05 g/Kg) amount of sodium (Lam & De Lumen, 2003), which is good for health because of the relationship that low sodium diets has to hypertension in humans (Apata & Ologhobo, 1994).

Studies have shown that the gross energy value of the BGN seeds is greater than that of common pulses such as cowpea and lentil and its protein content is higher in the essential amino acids particularly methionine compared with other legumes (Ijarotimi & Esho, 2009).

Legumes seeds are excellent sources of folate, which, in addition to being an essential nutrient, is thought to reduce the risk of neural tube defects (Lam & De Lumen 2003). Beans are also good sources of thiamine and pantothenic acid. On average 100 g of legume seeds provide 23% of the nicotinic acid, 50% of the thiamine, 15% riboflavin, 20% of the vitamin B_6, 195% of the folate, and 30% of the pantothenic acid requirement of an adult (based on US RDAs (Anon, 2010b)). It has been recognised that legumes are functional foods that both promote good health and have therapeutic properties (Amarowicz & Pegg, 2008). Legumes have shown to have low glycemic index, hypocholesterolaemic effect, breast cancer prevention and health benefits with respect to cardiovascular diseases and bone health (Awaisheh *et al.,* 2005). Hence, BGN is a potential functional food ingredient, its milk and probiotic beverage will likely provide good nutrition (protein, vitamin, polysaccharides, antioxidants, fat, minerals) inherent in the seeds.

2.5 Bioactive compounds and pharmaceutical value of legumes and BGN

Antioxidants are principal ingredients that protect food quality by retarding oxidative breakdown of lipids (White & Xing, 2001), the human body cells and tissues from the damaging effects of toxic molecules called free radicals (Adelakun *et al*., 2009). Most legumes contain some antioxidants (Lam & De Lumen, 2003). Typical compounds that possess antioxidant activity includes phenols, phenolic acids and derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments and sterols. Phenolic compounds are widely distributed in legumes. Chlorogenic, isochlorogenic, caffeic, ferulic, p-coumaric, syringic, vanillic and p-hydroxybenzoic acids are commonly present in legumes (White & Xing, 2001).

Legumes contain a number of bioactive substances including phenolics that can diminish protein digestibility and mineral bioavailability. Protease inhibitors from legumes can interfere with protein digestion, two types trypsin and chymotrypsin inhibitors have been identified in soybean (Lam & De Lumen 2003). Boiling dry beans generally reduces the protease inhibitors content by 80-90%. A high tannins concentration (4.5-15.0 mg/CE g) was observed for the black variant of BGN (Nti, 2009). Alain *et al.* (2007) reported improving the nutritional quality of BGN flours by eliminating anti-nutrients and concentrating most essential nutrients after treating the flours with 60% ethanol. Tannins are biological active compounds may have beneficial or adverse nutritional effects (Nti, 2009). Condensed tannins, the predominant phenolic compounds in legumes seeds, were widely found in lentil, pea, coloured and common beans (Nti, 2009). Tannins are located mainly in the testa and play an important role in defence system of seeds that are exposed to oxidative damage by many environments factors (Xu & Chang, 2007). Tannins concentration increase with seed coat colour intensity (Nti, 2009). Dehulling increased the protein content, reducing tannins content by up to 92% and improving the colour of bambara products, while heat treatment enhanced their aroma, taste and overall acceptability (Nti, 2009).

Phenolic compounds such as flavonoids, phenolic acids, lignans and tannins have antioxidant properties, and these are very important from nutritional and technological points of view. The antioxidant capacity of legumes depends on the biological varieties of the plant (Amarowicz & Pegg, 2008). Phenolics are natural antioxidants which represent an important group of bioactive compounds in foods which may prevent the development of many diseases including atherosclerosis and cancer (Onyilagha *et al.,* 2009). They also act as protective factors against oxidative damage and possess antimutagenic activity. Tsuda *et al.* (1994) reported screening of antioxidative activity of edible pulses; cyanidin 3-O-β-D-glucoside an anthocyanin pigment isolated from red bean (*P. vulgaris* L.) showed strong antioxidative activity.

Anthocyanins are water soluble pigments that are responsible for the red, blue, and purple colours in most flowers, seeds and fruits (Da Costa *et al.,* 2000). Tsuda *et al.* (1994) examined the antioxidative role of the pigments in the seed coat of pea bean. The pigment anthocyanins are glycosylated derivatives of the 3, 5, 7, 3`-tetrahydroxyflavylium cation and are classified as flavonoids. Their

beneficial pharmacological activities and possible health benefits of the anthocyanins confer a distinct advantage for their use (Da Costa *et al.,* 2000). Colouration of the different varieties showed marked variation, this was attributed to the cultivation region and the harvest time and varieties (Nti, 2009). Anthocyanins were observed to possess, known biological activities (Pale *et al.*, 1997). Pale *et al*. (1997) identified 3-β-O-monoglucoside of cyanidin, petunidin and malvidin from the seed coat pigments of BGN seeds. Tsuda *et al*. (1994) stated that, 3-O-β-D-glucoside is found widely in many seeds, and it may play an important role as a dietary antioxidant after ingestion in the prevention of lipid peroxidation of cell membranes induced by active oxygen radicals in the living systems.

Cowpeas contain bioactive antioxidants such as tocopherols, Vitamin C, carotenoids and phenolic compounds. Flavanoids and proanthocyanidins function as antioxidant (Onyilagha *et al.,* 2009). Kaempferol, km-3-O-rutinoside; km-7-O-rhamnoside; km-3-O-glucoside and km-3-O-glucosyl-7-rhamnoside are antioxidants isolated from BGN (Onyilagha *et al*., 2009). Red peanut skins were found to contain 7-9% procyanidins with 50% of these as low as low molecular weight phenolic oligomers. The low molecular weight phenolics were mainly flavan-3ols of catechin and epicatechin (White & Zing 2001). Tocopherols have long tradition of being hydrogen-donating antioxidants, peanut oil was found to contain between 350 and 650 ppm tocopherols (White & Zing 2001). Soybean concentrates and aqueous extracts of soybeans contain isoflavones and phenolic acids as the main antioxidants, organic solvents extracts contain tocopherol, sterol, phospholipids and other flavonoids while proteins hydrolysates contain antioxidants, amino acids and peptides (White & Zing 2001). Antioxidants from nuts are generally localised in the seed coat with lower amounts in the cotyledons. Red and black seed coat of BGN exhibited strong antioxidant activity but their antioxidative mechanisms were different (Onyilagha *et al.,* 2009).

The addition of antioxidants has become popular as a means of increasing shelf life of food and improving the stability of lipids and lipids containing food by prevention of loss of sensory and nutritional quality. In living systems antioxidants may be effective against oxidative damage. Phenolics antioxidants might be of interest as a native ingredient for example skin protecting additive in dermatology (Peschel *et al*., 2006). Synthetic antioxidants are suspected to be carcinogenic;

which has led to the multiple investigations in the field of natural antioxidants (Peschel *et al.,* 2006) therefore the importance of natural antioxidants has increased greatly, thus making the BGN seed coat a potential source (Onyilagha *et al.*, 2009).

Perhaps the bioactive content of BGN may explain some medicinal uses reported in some countries for BGN. Leaf preparations are applied to abscesses and infected wounds; sap from bambara leaves is applied to the eyes to treat epilepsy; roots are sometimes taken as an aphrodisiac and pounded seeds are mixed with water and used to treat cataracts in Senegal (Brink & Belay, 2006). The Igbos in Nigeria use the plant to treat venereal diseases (Brink & Belay, 2006). The Lio tribe in Kenya use BGN for treating diarrhoea, as water from boiled maize and BGN is drunk (Goli *et al.,* 1997). Extracts from leaves mixed with those of *Lanfana triflia* L. make a solution that can be used to wash live stock or as an insecticide (Mandawire, 2007). The black seeded variety has a reputation of being a treatment for impotence in Botswana (NRC, 1996). Chewing and swallowing raw seeds is believed to check nausea and vomiting and often used to treat morning sickness in pregnant South African women (Swanevelder, 1998). It is expected that a probiotic beverage from BGN will confer all the nutritional benefits especially antioxidative activity to the consumer.

2.6 Nutritional and economic value of milk from plants and vegetables

Vegetables do not produce literal milk, like a cow, however there are products made from oil seeds, legumes and cereals that resemble cow milk in appearance and nutrition. Tradition and economic reasons that limit the use of dairy products promote the idea of reducing dairy products as vehicles of the probiotic agents or even replacing milk from other media, such as cereals, fruits and vegetables (Granato *et al.,* 2010). Most prominent of these is soybean milk, its nutritional content is very close to that of cow milk (Prado *et al*., 2008). It can be coagulated to make cheese and fermented to make yoghurt. The production of vegetable milk using legumes and oil seeds is an old technology. However, the technology has been improved to include development of vegetable alternatives to dairy milk, especially in the formulation of infant foods because they are high in protein, mineral and vitamins. Legumes that have been used in vegetable milk production include soybean, cowpeas, winged bean, groundnut and melon seeds (Rivera-

Espinoza & Gallardo-Navarro, 2010); chickpea, pigeon peas, black graw, mung beans, coconut, lupin, peanut, and sunflower seeds (Quasem *et al.,* 2009) and BGN (Brough *et al.,* 1993). Growing awareness of the nutritional benefits of plant based foods by health conscience consumers, religious reasons, vegetarianism, cholesterol free, lactose free and dairy free diets has led to the increased interest in production of vegetable milk (Blandino *et al.,* 2003). A lot of attention has been given to soybean milk and its protein isolate beverage since they are considered to be nutritious and healthy (Quasem *et al*., 2009). Consequently soybean and peanuts have been used in a variety of milk based products including coffee creamers and chocolate milk drink (Aidoo *et al*., 2010). Legumes and oil seeds have characteristics that make it convenient to combine two or more to obtain an acceptable product. Vegetable milk made from peanut and cowpea blends could be dehydrated to produce an inexpensive dry milk powder (Aidoo *et al.,* 2010).

Vegetable milk and vegetable milk products have nutritional benefits for young and old people because of their extreme richness in protein, minerals, essential fatty acids, which are considered to be highly valuable for human nutrition (Brough *et al*., 1993; Wang *et al*., 2007). It is also suitable for both religious (vegetarians) and health (children who are allergic to cow milk protein, people on cholesterol free, lactose free, and dairy free diets) reasons (Granato *et al.,* 2010). The current interest in vegetable milk has been motivated by dairy and dairy products being priced too high for the low income earners (Lawal *et al*., 2007). Peanut milk is a low cost edible product with a high nutritional value. In this regard researchers have focused on products, resulting from fermentation such as yoghurt, buttermilk and ripened cheese analogues (Lawal *et al*., 2007). Lactose intolerance, cholesterol content and allergenic milk proteins are the major drawbacks related to the intake of dairy products, which makes the development of new probiotic foods essential (Granato *et al.,* 2010).

Fermented foods can be included in the category of functional foods, owing to their calcium content and other health promoting components (Prado *et al*., 2008). There is a greater interest in the potential beneficial effects of the fermented milk on health resulting in the increase of available varieties and amount consumed around the world. Dairy products have been used traditionally as vehicles of probiotics in humans (Granato *et al.,* 2010). Nothing is known about

BGN milk (BGNM) as a probiotic beverage. BGNM has a potential for use in probiotic beverage owing to its similarity to soybean milk.

2.7 Technology of vegetable milk production

Production of vegetable milk involves the extraction of the juice. This can be done in a number of ways depending on the type of the seed of interest and the test objective. The following paragraphs are a summary of procedures used by other researchers. Peanuts, being high in energy and cowpeas being low, the two were combined in specific ratio (1:2), slurred in a blender. The slurry was further milled using a colloid mill to obtain a smooth, fine, homogeneous milk. The milk was then dehydrated. The milk flakes were then milled to a fine powder with a hammer mill (Aidoo *et al*., 2010). Peanut milk was produced by soaking and grinding the seeds with water to get slurry, and then subjected to filtration. Alternatively it was also produced by grinding unsoaked roasted peanut, raw full fat or partially defatted peanuts, to form flour to which water was added to make an emulsion (Aidoo *et al.,* 2010). Peanut protein isolates can be used to supplement animal milk.

Akinyele and Akinlosotu (1991) described the extraction of cowpea milk. The cowpea seeds were soaked overnight, dehulled and resoaked for 24 hours. The soaked seeds were then divided into two, half was used to prepare the raw milk direct, and the other half was toasted to prepare pre-treated milk. The raw milk was prepared by homogenising one portion of the soaked beans with hot water, initially in a Kenwood food processor to reduce the size, followed by high speed mixing using a Silverson food mixer to ensure a fine suspension of beans and water. Pre-treated milk was produced by first lightly toasting the beans prior to homogenising one portion with hot water, other portion with cold water, until the colour was slightly translucent. After homogenisation, the milk was strained through a muslin cloth.

Nnam (1997) described the production of vegetable milk where a fermentation step was included. Cowpea seeds were dehulled, and milled using a laboratory hammer mill to fine flour. The flour was mixed with deionised water at a ratio of 1:4 and allowed to ferment by the micro flora present in the paste for 24 hours at an average room temperature. The fermented product was freeze dried to a 96% dry matter. The dried flour was milled in the laboratory hammer mill to

fine powder. The flour was then mixed with deionised water in a Silverson food mixer. The slurry was then filtered through a double muslin cloth to yield the test emulsion which was centrifuged and autoclaved.

Legumes are unpopular to some people because of their characteristic beany flavour (Alobo, 1999). Advances in fermented products manufacture revealed that hexanal, which is one of the compounds responsible for the unwanted beany flavour, disappears completely as a result of fermentation (Nnam, 1997; Champagne, & Gardner, 2008). Experience of milk production from cowpea and soybean which are closely related to BGN, has shown that in order to remove the beany taste, associated with vegetable milk, it is necessary to heat treat the seeds prior to homogenisation (Brough *et al*.,1993). Quasem *et al*. (2009) prepared sesame seed milk from decorticated seeds. The seeds were mixed with tap water and ground, then homogenised, one portion was pasteurised while the other was sterilised. The study revealed the possibility of producing sesame milk with reduced beany flavour.

The production of BGNM has to be done in such a way that the non-fat solids meet the legal standards for the coagulum to be acceptable to the consumer. Best quality of yoghurt is obtained when the milk has 15-16 g/100 g solids (Tamime & Robinson, 1999). Low protein content in the milk might require fortification with non-fat solids, which will improve the consistency of the final product. Hydrocolloids are normally used as stabilisers; they bind water and promote the increase in viscosity. The gel strength and syneresis are influenced by the method of fortification; the best method for BGNM needs to be investigated. Stabilisation of the BGNM is another step that requires attention, the right temperature, pressure and time must be employed so as to get an acceptable final product (Tamime & Robinson, 1999).

2.8 Physiology and benefits of probiotics in human diet

Probiotics are defined as 'live organisms which when administered in adequate amounts, confer a health benefit on the host' (Champagne & Gardner, 2008; Prado *et al.*, 2008). Probiotics are a major topic of lactic acid bacteria (LAB), *Lactobacillus* and *Bifidobacterium* (Alander *et al*., 1999). Interest has grown in the commercial utilisation of *Lactobacillus* strain, owing to its health promoting benefits.

The microorganisms must be alive and present in high numbers, generally more than 10^9 cells per daily dosage (Shima *et al.,* 2006). A total of 78% of current probiotic sales in the world are delivered through yoghurt (Granato *et al.,* 2010). Fruit juices desserts and cereal-based products featuring probiotics are also suitable media for delivering probiotics (Ranadheera *et al.,* 2010). Lavermicocca (2006) mentioned that in the 1900s a Russian scientist Metchnikoff concluded that fermented foods prolong life, he also reported that daily intake of probiotics contribute to improving and maintaining well balanced intestinal flora and it prevents gastrointestinal disorders. The genera *Lactobacillus* and *Bifidobacterium* are normally used as probiotics (Champagne *et al.,* 2009).

LAB belongs to a group of gram-positive anaerobic bacteria that excrete lactic acid as their main fermentation product into the culture medium (Isolauri *et al.,* 2000). LABS were among the first organisms to be used in food manufacturing, they contribute significantly to human health and nutrition (Furet *et al.,* 2004). Today they play crucial roles in the manufacturing of fermented milk products, vegetable, fruit, cereals (Granato *et al.,* 2010); meat, wine (Furet *et al.,* 2004) and vegetable milk (Wang *et al*., 2007). LAB are also commensally inhabitants of the gastrointestinal tract in humans and animals where they contribute to the complex interactions between the intestinal microbiota and the host (Furet *et al.,* 2004).

As probiotics, LAB have shown that they may present significant beneficial clinical effects in preventing and treating diarrhoea, and in improving the digestion of lactose by lactase deficient individuals, preventing or treating certain allergies (Isolauri *et al.,* 2000) and inflammatory bowel diseases and cholesterol lowering (Scheinbach, 1998; Kaur & Kapoor, 2002). Figure 2.4 summarises the benefits of probiotics in human nutrition. However, in order to exert their beneficial effects in the host, bacteria have to be alive during their transit time through the gastric juice, in the stomach, when they reach the small intestine and colon (Rivera-Espinaza & Gallardo-Navarro, 2010). Although recently there are convincing data on beneficial immunological effects derived from dead cells, Rivera-Espinaza & Gallardo-Navarro (2010) reported that best effect is achieved when the microorganisms colonise the intestinal epithelium since they can affect the intestinal immune system, displace enteric pathogens, provide antimutagens

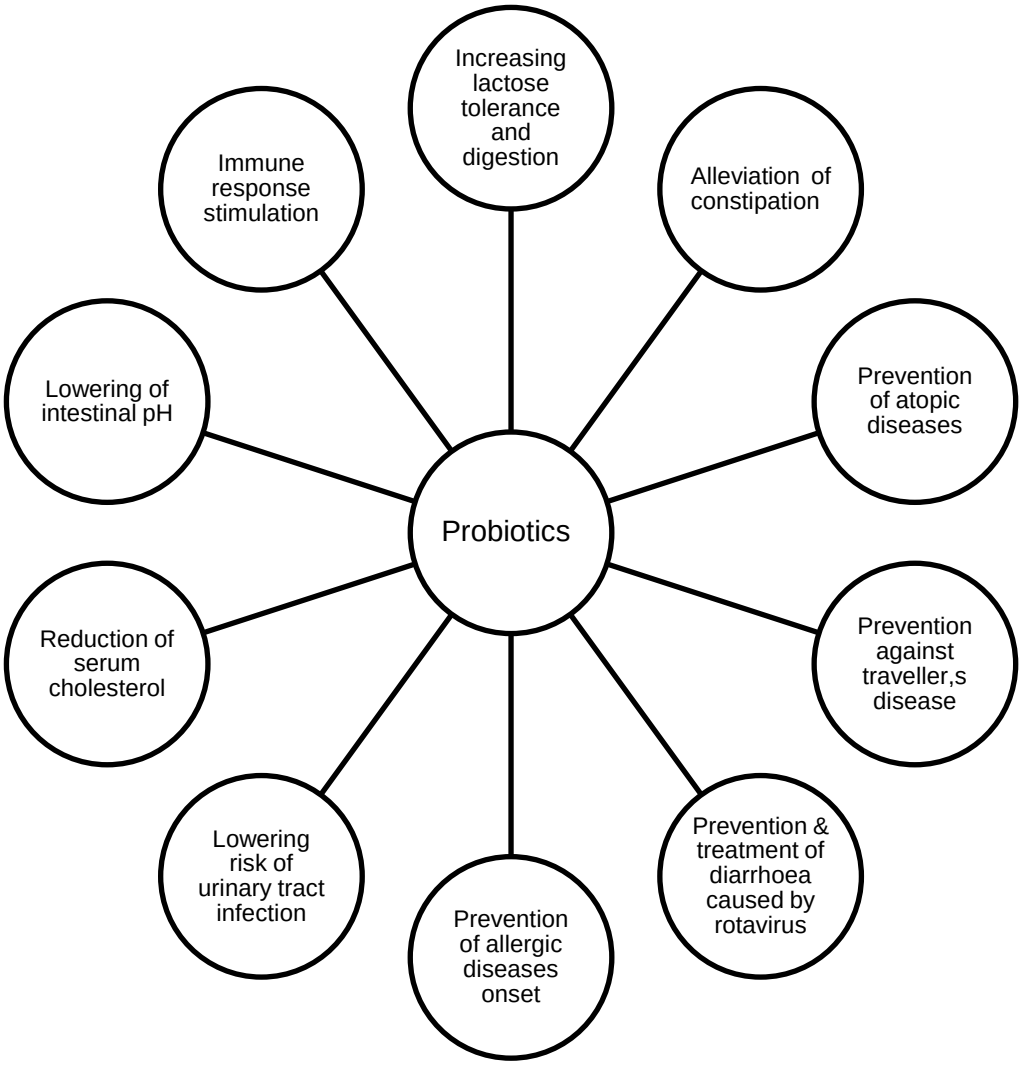


Figure 2.4 Some of the benefits of probiotics. Adapted from Lavericocca, 2006; Shima *et al.,* 2006 Prado *et al,* 2008; Champagne & Gardner, 2008; Ranadheera *et al.,* 2010.

and antioxidants, and possible other effects by cell signalling. It is advisable to note that the health benefits imparted by probiotics are strain specific, no strain will provide all proposed benefits (Gorbach, 2002). Soybean yoghurt is usually made using the normal yoghurt cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus,* this can hold true for BGNM. Nevertheless, selection of probiotics which survive and thrive well in the BGNM must be investigated.

Probiotics in the form of fermented milk products have been consumed for centuries (Lavermicocca, 2006). In this century various health benefits have been reported to result from consumption of foods containing live microorganisms, particularly LAB (Scheinbach, 1998). *Lactobacilli* occur naturally in the human intestine (Kneifel *et al*., 1993; Shimakawa *et al.,* 2003); therefore they are preferentially developed for commercial use as probiotics. Functional properties of probiotics are the production of antimicrobial compounds, and the ability to modulate immune response and adhesion to the gut tissue (Heller, 2001). Antagonisation of pathogens by probiotics, involve production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins (Lavermicocca, 2006). The complex composition of the intestinal flora is relatively stable in healthy human beings (Awaisheh *et al.,* 2005), any disturbance results in changes in the intestinal flora, which consequently allows undesirable microorganisms to dominate in the intestine and as a result leads to infectious diseases (Oliveria *et al.,* 2001). Factors that influence microbiota population include contributions from host physiology, endogenous nutrients (Adolfsson *et al.*, 2004), intestinal pH, microbial interactions, environmental temperature (Oliveria *et al*., 2001), peristalsis, bile acids, host secretions, immune responses, drug therapy, bacterial mucosal receptors (Park & Oh, 2007), aging, stress, diet (Rivera-Espinaza & Gallardo-Navarro, 2010), bacterial contamination and constipation (Gismondo *et al.,* 1999). The association between stress and various gastrointestinal diseases, including functional bowel disorders, inflammatory bowel disease, peptic ulcer disease and gastro oesophageal reflux disease, is being actively investigated (Heller, 2001).

Beneficial health promoting effects are strain specific, therefore when selecting new candidates for probiotic application functionality and technological characteristics should be tested separately for each strain. Knowledge of the mechanism underlying these health benefits is limited. However, the mode of

action has been reported by Bao *et al*. (2010) as production of antimicrobial substances, competition of nutrients, competitive exclusion of pathogen binding and modulation of the immune system.

The inherent presence of antioxidants, chemical and nutritional composition of BGN makes its milk a potential ingredient for fermentation by LAB as a beverage.

2.9 Gastric survival and growth

Probiotics need to be viable within the gastrointestinal tract therefore they must survive the transit through the adverse conditions (bile salts, pancreatin) of the small intestine and reach the colon in large quantities to facilitate colonisation and thus exert beneficial effects on the host (Lian *et al.,* 2003). *Lactobacillus* strains which are mostly delivered in food systems must overcome physical and chemical barriers in the gastrointestinal tract especially acid and bile (Molly *et al.,* 1996). Probiotics need to possess the ability to survive in the product with sufficient number during production and storage (Granato *et al.,* 2010).

Tolerance to gastric juice, bile and inhibition of harmful pathogenic bacteria can be used as screening tests for probiotic ability of microorganisms (Kneifel *et al.,* 1993). When probiotics enter the large intestine they need to compete for nutrients and ecological colonisation with previously established micro flora (Pinto et al., 2006). *Lactobacillus* strains which are mostly delivered in a food system must overcome physical and chemical barriers in the GI especially acid and bile. Probiotics need to possess the ability to survive in the product with sufficient numbers during production and storage. *Lactobacillus casei* Zhang is a potential probiotic isolated from koumiss with high acid and bile salts resistance, persistence of gastrointestinal tract transportation, cholesterol reducing and antimicrobial activities (Tuomola *et al*., 2001; Bao, *et al.,* 2010).

Gastrointestinal models simulating the dynamic physiochemical process of the gastrointestinal tract (Molly *et al*., 1996) have been used to study the growth and survival of probiotics. The physiochemical parameters include pH, concentration of gastric juice, small intestine enzymes, concentration of bile salts and the kinetics of passage of chime through the stomach and the intestine (Alander *et al.,* 1996). *In vitro* model simulating the ecology of the human colon offer possibilities of studying the interactions of probiotic strains with normal flora,

for example metabolic activity, production of volatile fatty acids gases, microbial enzymes and bacteriocin (Alander *et al*., 1996).

In order to screen for the probiotics' capacity of transit tolerance to the upper gastrointestinal tract, pH of the stomach could be as low as 1.5, to provide an effective barrier against entry of bacteria into the intestinal tract (Lankaputhra & Shah, 1995) or as high as 6 or above after food intake (Goldin & Gorbash, 1992), but generally ranges from 2.5 to 3.5 (Goldin & Gorbash, 1992). The nature of food affect the transit time through the stomach between 2 to 4 hours (Alander *et al*. 1996). However, liquids empty faster than solids and only take about 20 minutes to pass through the stomach (Mathara *et al*., 2008). There are no agreed rules on screening but pH 1 to 5 has been used to screen *in vitro* the acid, however studies are usually done using a transit time generally between 1 to 4 hours (Goldin & Gorbash, 1992) pH 8. Bile salts resistant LAB can be selected by testing their survival in the presence of bile salts and their growth in selective medium containing various levels of bile. A concentration of 0.15 to 0.3 of bile has been recommended as a suitable concentration of selection probiotics bacteria for human use (Goldin & Gorbash, 1992).

Non-viable probiotics are ones which are not capable of living, developing or germination under favourable conditions. Lack of viability could be as a result of high temperature treatment, chemical and when the bacteria reach the lag phase of their growth. In food regulation viability of bacteria is emphasised (Anon, 2010b). Studies have been carried out on viable bacteria where non-viable bacteria were used as the placebo, but had some probiotic activity (Ouwehand & Salminen, 1998). Non-viable probiotics can be used to expand the potential use of probiotics to areas where strict handling conditions cannot be met for example in developing countries, they also have economic advantage in terms of longer shelf life, reduced requirements for refrigerated storage, easier handling and transportation.

2.10 Yoghurt as probiotic beverage

Microbiologically yoghurt is an end product of a controlled fermentation of high solids whole milk with a symbiotic mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Vendamutha, 1982). The South African legislation defines yoghurt as the product obtained from pasteurised milk

or reconstituted milk which has been inoculated with a yoghurt culture and which is allowed to ferment under controlled conditions. Milk is the normal mammary gland secretion obtained from lactating cows of the bovine species, goats or sheep (Anon, 2010c). Nevertheless, Shimakawa *et al*. (2003) argues that soybean milk can be treated in the same way as cow`s milk when making soy yoghurt.

Yoghurt-like products are available, they are expected to exert a beneficial effect on the health status of the consumer as they contain LAB isolated from the human intestine which are claimed to have probiotic properties (Granato *et al*., 2010). Application of LAB cultures of human origin in the manufacture of fermented milk products is not a new concept (Schillinger *et al.,* 1999). Fermented dairy products are increasing in popularity as convenient, nutritious, stable, natural and health food (Granato *et al.,* 2010). *Lactobacillus acidophilus* is a member of the normal flora of humans.

Strain selection involve ascertaining that the bacteria must be regarded as Generally Recognised As Safe (GRAS), therefore *Lactobacillus* species, dairy *Propionbacteria* have been used as starter culture in the dairy industry for a long time, and are considered safe for human consumption (Schillinger *et al* 1999). Figure 2.5 shows some of the selection criteria used for characterising bacteria into probiotic strains (Kneifel *et al.,* 1993; Schillinger *et al*., 1999; Olivera *et al*., 2001; Shimakawa *et al.,* 2003).

Lactobacillus acidophilus and *Bifidobacterium bifidum* are the two most important strains to look for in probiotic beverages, but there are more than 400 strains of beneficial probiotics (Shimakawa *et al.,* 2003). *Lactobacillus acidophilus* bacteria lives in human oral cavity, small intestine, and the vaginal epithelium, where it is thought to play beneficial roles (Ranadheera *et al.,* 2010), and helps to digest other nutrients, including proteins and milk, as well as producing natural antibiotics that help fight harmful bacteria and can lower low density lipoprotein (LDL), the so called “bad cholesterol” (Olivera *et al*., 2001). *Bifidobacterium* is the good gut flora found in the large intestine which aids in the production of B vitamins, increases acidity to help destroy harmful bacteria, improves bowel function and eliminates bowel toxins (Kneifel *et al.,* 1993; Shimakawa *et al.,* 2003).

Good quality yoghurt is obtained when the yoghurt cultures are used in the 1:1 ratio, at a temperature range of 35-45^{o}C (Vendamutha, 1982; Kneifel *et al.,* 1993).

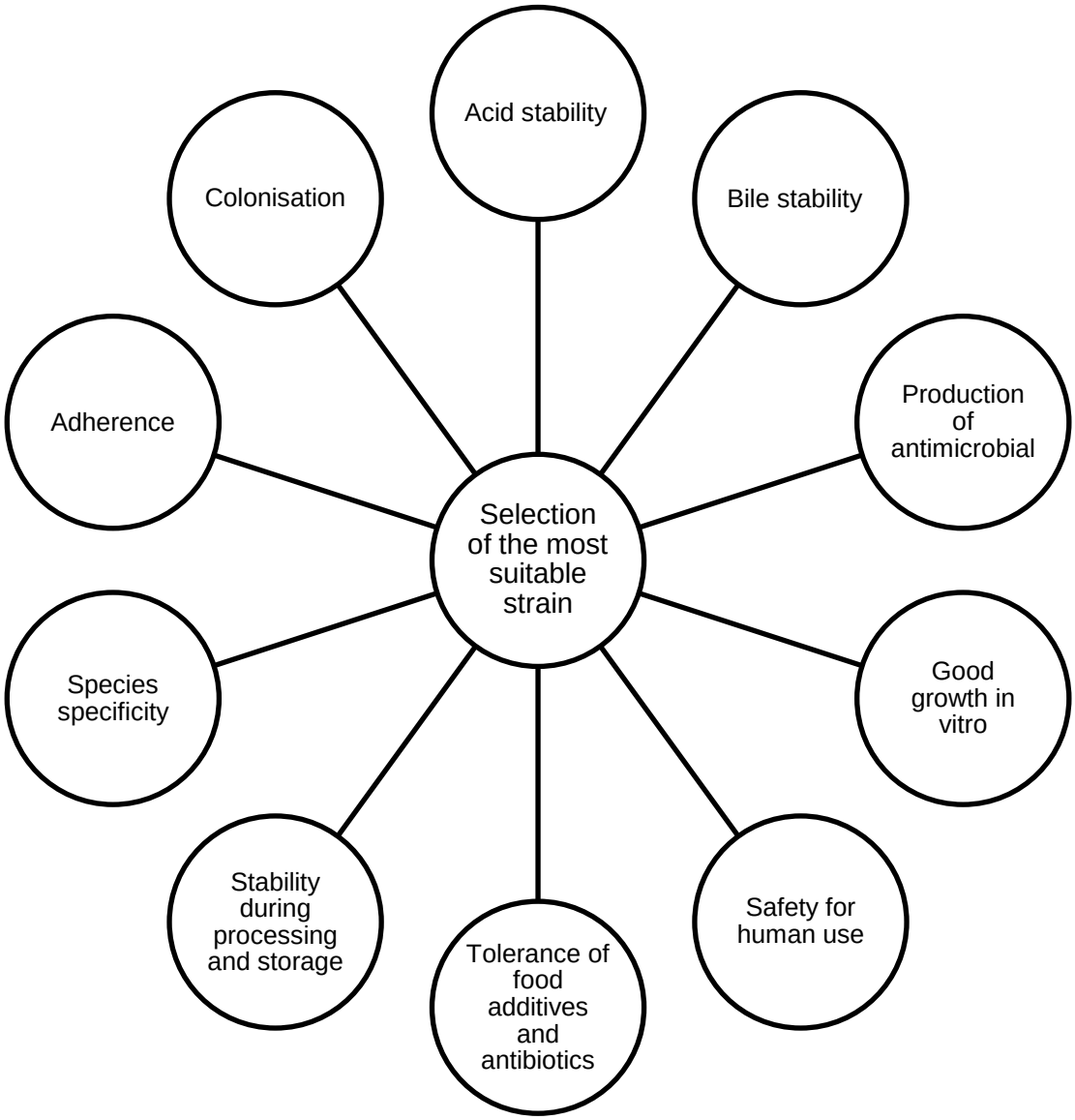


Figure 2.5 Selection criteria for probiotic strains. Adapted from Kneifel *et al.,* 1993; Schillinger, 1999; Olivera *et al*., 2001; Shimakawa *et al.,* 2003.

Streptococcus and *Lactobacillus'* role in yoghurt manufacture can be summarized as milk acidification and synthesis of aromatic compounds (Kneifel *et al.,* 1993; Shimakawa *et al.,* 2003). In both *Streptococcus* and *Lactobacillus*, lactose is used via the Embden-Meyerhof-Parnas pathway in which the pyruvate is reduced to lactic acid (Serra *et al.,* 2009).

Flavour compounds that contribute to the final aroma of yoghurt may be divided into four categories: non-volatile acids (lactic or pyruvic or acetic); volatile acids (butyric or acetic); carbonyl compounds (acetaldehyde or diacetyl) and miscellaneous compounds (amino acids or products formed by thermal degradation). The acidic and organoleptic properties of fermented foods result from the metabolic activity of the microorganisms, but also aroma and flavour characteristics directly or indirectly by the fermenting organisms (Serra *et al.,* 2009).

Quantitative determination of organic acids is important for monitoring bacterial growth and activity (Yoon *et al.,* 2004; Serra *et al.,* 2009). Organic acids are particularly important in fermented products such as yoghurt, since they play an important role as preservatives, but also because they strongly contribute to the sensory characteristic of the product. The presence of organic acids in dairy products may be due to several reasons, including bovine metabolic processes, bacterial growth or hydrolysis of milk fat as reported by Serra *et al.* (2009).

Klaver and Van der Meer (1993) reported that one of the most constraining drawbacks associated with the use of dairy cultures in fermented milk products is the lack of acid tolerance of some species and strains. The pH of yoghurt may drop to a level as low as 3.6 which may result in the growth inhibition of *Bifidobacteria* since their growth is retarded below pH 5.0. Growth and progression of *Bifidobacterium* species in yoghurt are suppressed due to different rates of multiplication of bacteria strains present during fermentation. Inoculation temperature is also an important factor; lower incubation temperatures (37-40^{o}C) will favour the growth rate and survival of probiotic species (Kneifel *et al*., 1993).

The body and texture of the end product is affected by the fat content; non-fat solids; concentration in the mix; stabilizers used; heat treatment of the mix; concentration of the proteins; concentration of calcium and magnesium and the starter cultures used (Vendamutha, 1982). Cultures most suitable for the BGN probiotic beverage needs to be investigated, but the soybean yoghurt already in

the market is an indication that a BGN probiotic beverage (BGNMPB) can be manufactured.

2.11 Adaptation of the vegetable milk for survival of probiotics

Vegetable milk being different from the dairy milk is treated in such a way to make it more favourable to yield a product similar to their dairy counterparts (Granato *et al.,* 2010). The technological advances have made possible the alteration of some structural characteristics of fruit and vegetables matrices making them ideal substrates for probiotic cultures (Granato *et al.,* 2010). Such modifications include among others pH and fortification of culture media. BGN milk contain beneficial nutrients, such as minerals, amino acids (Uvere *et al,* 1999), vitamins and bioactive substances (Tsuda *et al.,* 1994; Pale *et al.,* 1997) while lacking the dairy allergy which will encourage consumption by certain segments of the population (Quasem *et al.,* 2009). Lactose and/or milk proteins are added to soybean milk during yoghurt production for culture activity enhancement. When culture stimulants are not added the fermentation process is prolonged (Nsofor *et al.,* 1996) or starter concentration has to be increased (Tamime & Robinson, 1999). It will be interesting to ascertain if BGNM will require culture activity enhancement.

Ideal vegetable milk should be inoculated with LAB that are capable of fermenting galactooligosaccharides such as raffinose and stachyose in soy beans. In this regard Nsofor *et al.* (1996) evaluated acid production rates in soymilk with 1% sucrose and cow milk, their results indicated a non-dependence of culture on milk constituents. Non-dairy stimulants, however, may be needed to achieve high activity at low starter concentration (1-3%) normally used for yoghurt production (Granato *et al.,* 2010). Yeast extract has been shown to induce high activity in cheese starter culture, and may also stimulate vegetable milk starter culture (Uvere *et al.,* 1999). Several sub culturing stages in vegetable milk during its development may induce synthesis of enzyme that hydrolyses soybean oligosaccharide which causes flatulence in humans (Quasem *et al.,* 2009). Repeated sub-culturing may lead to great reduction of starchyose concentration in soy yoghurt (Nsofor *et al.,* 1996).

Active microorganisms interact with their environment by exchanging components of the medium for metabolic products, therefore chemical composition of the BGNM is very important for the metabolic activities of the product (Granato

et al., 2010). The type and quantity of carbohydrates available, and degree of hydrolysis of the milk proteins which in turn determines the availability of essential amino acids, plays a significant role during the fermentation process (Heller, 2001). Proteolytic and lipolytic properties of probiotics may be important for further degradation of proteins and lipids which affect the taste and flavour of the end product (Heller, 2001).

There are wide varieties of traditional non-dairy foods developed around the world many of which are non-alcoholic beverages (Granato *et al.,* 2010). *Boza* is a cold beverage made from fermented cereal; *Bushera* is a cereal based beverage prepared in the Western highlands of Uganda; *Mahewu* is a sour beverage made from corn meal; *Pozol* is a refreshing beverage made with cocoa and cornmeal; *Togwa* is a starch-saccharides traditional beverage that has been used as a probiotic medium (Granato *et al.,* 2010). Other non-dairy products available in the market place are effervescent tablets, chewable tablets and drinking straws (Granato *et al.,* 2010). Farnworth *et al.* (2007) pointed out the potential of root crops, legumes, shrimp, and cassava, diverse types of vegetable flours, fish, fruit seeds, meats and fungi based substrates as well as milk from other varieties of animals for the development of new probiotic foods (Granato *et al,* 2010).

Food is common delivery system for probiotics bacteria. Food and food ingredients have been shown to protect probiotics from harsh gastrointestinal tract conditions (gastric juice and bile) (Mathara *et al*., 2008). Milk has been reported to increase the viability of acid sensitive *Lactobacillus* and *Bifidobacterium* strain during simulated gastric juice transit. Protective effect may be due to the increase of gut pH after milk addition (Conway *et al.,* 1987). Amylose maize starch granules at pH 3.5 have been found to increase the viability of the more acid sensitive *Bifidobacterium* strain (Wang *et al.,* 1999). Probiotics viability in the food matrix depends on factors, such as pH, storage temperature, oxygen levels, and presence of competing microorganisms and inhibitors (Granato *et al.,* 2010). It is significant that the BGN beverage formulation maintains the activity and viability of the probiotic for an extended period of time (Granato *et al.,* 2010)

LAB obtain energy only from the metabolism of sugars, thus they are restricted to environments in which sugars are present. LAB has a very limited capacity to synthesize amino acids using inorganic nitrogen sources; they are therefore dependent on preformed amino acids in the growth medium as a source

of nitrogen (Serra *et al.,* 2009). The inherent presence of antioxidants, chemical and nutritional composition of BGN makes its milk a potential ingredient for fermentation by LAB as a beverage.

2.12 Application of probiotic beverage technology to bambara groundnut milk

The technology involved in making probiotic soybean milk beverage, can be adapted for BGN milk perhaps with some modification to cater for the differences in physical properties. Amarteifio and Moholo (2004) reported the chemical similarities between soybean and BGN; hence the possibility of making a probiotic beverage from BGN.

Champagne *et al.* (2009) stated that probiotics generally do not grow rapidly in cow's milk, hence in yoghurt manufacture; they do not attain as high numbers as the starter cultures. Farnworth *et al.* (2007) studies indicated that soy is a good substrate for *Lactobacillus,* the final pH declined faster in the soy beverage than in the cow`s milk which might economising on production time. Perhaps, BGN milk can be good alternative thereby reducing quantity of inoculation starter culture, incubation time resulting to a better product in terms of high bacteria count. Kamaly (1997) noted that sucrose did not significantly affect the growth of *L. acidophilus* B-1910 and B-2092, but in soybean milk it gave a balanced taste of sweetness and sourness to the fermented product, in addition to stimulating bacterial growth which in turn, would increase titratable acidity. During soybean yoghurt production the milk was subjected to high temperature treatment before fermentation to destroy indigenous bacterial spores. However, high temperature could also have adverse effects on the quality of the fermented product. Hence, it is essential to find an optimum sterilisation condition for the BGNM. Kamaly (1997) indicated that fortifying soybean milk with certain carbohydrates, protein hydrolysates and amino acids seems appropriate to enhanced growth and acid development. Similarly, fortification of the fermenting BGNM can be investigated with an objective of making a nutritional and organoleptically superior product.

Conclusion

BGN is a nutrient dense legume that should attract the attention of scientists both in Africa and outside to tap on this richness. Being a drought tolerant crop and relative resistance to diseases and pests, BGN has the potential to (1) improve food security in many rural areas; (2) become a stable, low-cost and profitable food crop for Africa's small-scale farmers and (3) a new source of food ingredient for the food industry. Furthermore, being a crop growing in some of the most malnourished nations, it could be engaged in programs for fighting against malnutrition.

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CHAPTER 3

OPTIMISATION OF BAMBARA GROUNDNUT MILK PRODUCTION AND THE QUALITY AND SENSORY EVALUATION OF MILK FROM DIFFERENT VARIETIES

Abstract

The red variant of bambara groundnut (*Vigna subterranea)* (BGN) seeds from South Africa were milled, hydrated following a Central Composite Rotatable Design (CCRD) so as to optimise the hydration time (2-6 h) and temperature (25-35^{o}C) of BGN flour for optimum BGN milk (BGNM) production process. Reduced quadratic models for pH, linear model for total solids, two factor interaction model each for hue and lightness were effective in describing the effect of hydration time and temperature. Hydration time had a significant ($p < 0.05$) effect on the pH, stability, lightness, chroma and total solids, and no significant effect on the hue. The effect of temperature was significant ($p < 0.05$) for pH, lightness, chroma and total solids. The interaction of time and temperature had a significant ($p < 0.05$) effect on stability, lightness, and total solids, while the effect on the pH, chroma and hue was not significant. The optimal hydration time and temperature for optimum BGNM was estimated to be 2 h at 25^{o}C resulting in optimum BGNM with pH, hue and total solids of 6.52, 54^{o} and 2.6%, respectively. The results demonstrated the possibility of producing BGNM using hydrated BGN flour. BGNM prepared by hydrating BGN flour to water 1:10 ratio, from five varieties (black, red, brown, brown-eye, black-eye) were investigated for the effect of variety on pH, colour and antioxidative activity (radical scavenger 2,2-diphenyl-1-picrylhydrazyl (DPPH)) and consumer acceptability. Four of the milk (red, brown, brown-eye, black-eye) were subjected to sensory evaluation using 39 consumer panellists. The BGNM colour parameters ranged from 41.49 ± 0.27 to 54.34 ± 0.41 for lightness (L), 3.86 ± 0.66 to 9.70 ± 0.70 for chroma, 33.20 ± 24.63 to 54.07 ± 5.66 for hue, 1.39 ± 0.68 to 5.88 ± 1.14 for redness (a*), 3.56 ± 1.12 to

7.55 ± 1.64 for yellowness (b*), 0.39 ± 0.004 to 0.41 ± 0.002 for [absorbency (A)] DPPH and 6.56 ± 0.06 to 6.60 ± 0.006 for pH. Lightness, chroma, redness, yellowness and DPPH of the BGNM were significantly ($p \leq 0.05$) different from each other while hue and pH did not differ significantly. BGNM from black variety showed the most antioxidative capacity (0.39 A). Milk samples with high lightness (brown-eye, black-eye) were low in chroma, redness and yellowness. The colour differences between the BGNM ranged from 1.4 to 13.9 and were therefore perceivable to the consumers. The BGNM can be categorised into two groups on the basis of yellowness and hue. Black, brown and red BGNM were characterised by high hue (74.7^{o}, yellowish-red) but comparatively less in yellowness (b = 5.19); while brown-eye and black-eye were characterised by less hue (48.4^{o}, reddish-yellow) but comparatively high in yellowness (b = 5.44). The four BGNM samples were significantly different ($p < 0.05$) in appearance, colour, mouthfeel and overall acceptability but not in aroma and taste. BGNM could be classified into two groups based on appearance, colour and taste. BGNM from red and brown varieties were moderately undesirable in taste and moderately desirable both in appearance and colour, while those from brown-eye and black-eye were moderately undesirable both in taste, appearance and colour. The entire panellists less than 20 years old, 20-30 years (48.6%) and majority in age 31-40 (87.5%) equivalent of 53.2% of the panellists moderately desired the BGNM milk in appearance and colour but the taste is moderately undesirable. There is high preference for BGNM across all the age groups. The high preference for panellist aged <20 signals the high probability of acceptance of BGNM if commercialised and the potential of BGNM as an ingredient and a milk substitute.

3.1 Introduction

The BGN varieties consist of several genotypes which have different capacity to tolerate biotic and abiotic stress under a low input agricultural system (Zeven, 1998; Amadou *et al*., 2001; Basu *et al*., 2007). BGN varieties have recognizable morphological features like the testa colour and patterns which can be used for their identification (Massawe *et al*., 2002). Various testa patterns are found, including mottled, with or without hilum (eye) colouration, blotched or stripped, with or without hilum colouration (Goli *et al*., 1997).

On the basis of testa colour there are seven types of BGN varieties: (1) Black, usually small to medium-sized kernel, mainly one-seeded and matures early; (2) Red, late maturing, large kernels, however, it is prone to rotting onsite; (3) Cream/black-eye, a good yielder with large kernels; (4) Cream/brown-eye, a good yielder with moderate size of kernel; (5) Cream/no-eye, produces mainly one seed and lower yield with very small pods and kernels; (6) Speckled/flecked/spotted, kernels are small and pods are mainly one seeded with purple colour predominating; and (7) Brown, variation in colour between light and dark brown, kernels of medium to large size (Directorate Plant Production, 2011).

Growing awareness of the nutritional benefits of plant-based foods by health conscious consumers and consumers with special dietary requirements (religious taboos, vegetarians, cholesterol free, lactose free and dairy free) has led to the production of vegetable milk (Quasem *et al*., 2009; Boursier *et al*., 2012). Production of vegetable milk has been exploited by a number of researchers (Akinyele & Akinlosotu, 1991; Brough *et al*., 1993; Nnam, 1997; Alobo 1999; Lawal *et al*., 2007; Aidoo *et al*., 2010; Agundiade *et al*., 2011; Ukwuru & Ogbodo, 2011, Boursier *et al*., 2012). Milk extraction can be done on soaked seeds which are wet milled and then strained (Nsofor *et al*., 1996). The seeds can either be heat-treated (Akinyele & Akinlosotu, 1999), sprouted or even fermented (Nnam, 1997) before the milling, depending on the test objective. Brough *et al*. (1993) reported milk extraction from BGN using wet milling. No doubt there is huge energy consumption in wet milling. We thought that BGN from dry milled flour will save energy especially for commercial ventures.

The major legumes that have been used in vegetable milk production include soybeans, cowpeas, winged bean, groundnuts and melon seeds (Quasem *et al*., 2009). In the study by Agunbiade *et al.* (2011) BGNM was acceptable by the consumers and was recommended as weaning food. However, not much is known about BGNM from dry milled BGN flour. Furthermore, the effect of variety on some properties of the milk as well as its consumer acceptability is not documented. Producing BGNM from BGN flour will require a hydration process. The optimum time and temperature of BGN flour hydration for optimum BGNM is not documented.

The objectives of this study were to: (1) optimise BGNM production process from BGN flour and (2) investigate the effect of variety on the quality of BGNM in terms of colour, pH, antioxidative activity and consumer acceptability.

3.2 Materials and Methods

The overview of this chapter is depicted in Figure 3.1. The optimum hydration temperature and time for BGN flour was established. The determined temperature and time were used for the BGNM production from the five varieties black, brown, red, brown-eye and black-eye. The five milk samples were then assessed for colour, pH and antioxidative activity. However, only four milk varieties were assessed for consumer acceptability.

3.2.1 Source of BGN

Bambara groundnuts (BGN) were purchased from Thusano Products, Louis Trichardt, Limpopo, South Africa. The BGN seeds were sorted into five varieties, according to different testa and hilum colours as black, red, brown, black-eye and brown-eye.

3.2.2 Production of BGN flour

The red BGN seeds were milled into flour using a hammer mill (Bauermeister, Bauermeister Inc., Vernon Hills) with a sieve size 250 µm (No. 60), packed in clear plastic bag and stored in a refrigerator at 4-6^{o}C, till needed.

3.2.3 Optimisation of hydration time and temperature for optimal BGN milk

Figure 3.2 details the steps involved in the production of BGN milk (BGNM). Red BGN flour (25 g) was mixed with 250 mL distilled water (1:10), and allowed to hydrate in a water bath (Metter, Lasec, South Africa) for 2-6 h at 25-35^{o}C combination generated from the Central Composite Rotatable Design (CCRD) in Table 3.1. After hydration the slurry was filtered through muslin cloth with fine mesh size 707 µm (No. 25) folded four times to yield the BGN juice. The BGN juice was then homogenized (Silverson L4RT) for 5 minutes at 3000 rps, and allowed to stand for 5 minutes then decanted into a 500 mL schott bottle. Care was taken not to decant the starch sediments.

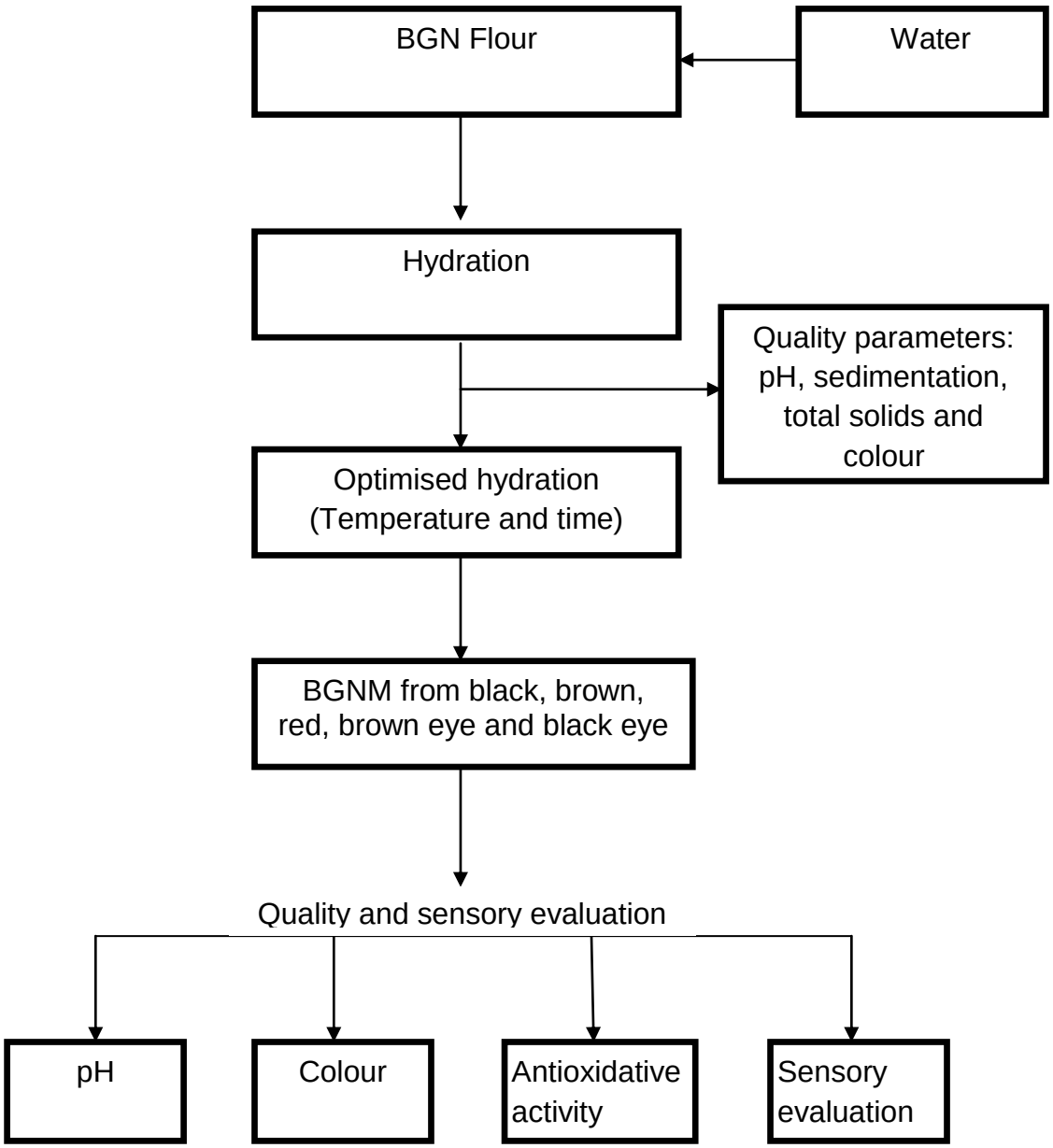

Figure 3.1 Overview of chapter three

BGN - bambara groundnuts

BGNM - bambara groundnut milk

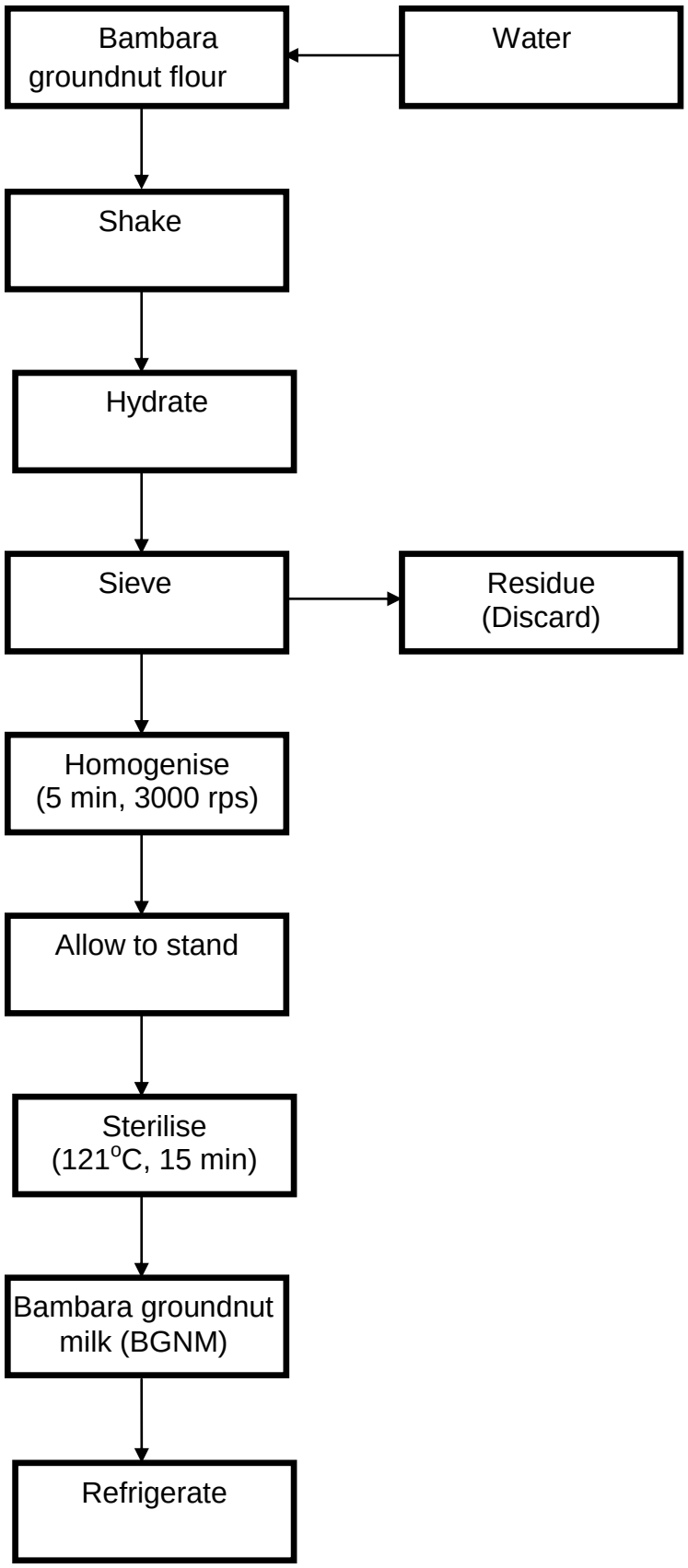

Figure 3.2 Production of bambara groundnut milk (BGNM)

Table 3.1 Process variables used in the CCRD for hydration time and temperature for BGNM[a]

Variable	Symbol	Coded Variable level (x_i)				
		-1.414 (α)	-1	0	+1	1.414 (+α)
Time (hr)	X_1	1.17	2	4	6	6.83
Temp (^oC)	X_2	22.93	25	30	35	37.07

[a]Transformation of coded variable (x_i) to uncoded variable (X_i) levels could be obtained from $X_1 = 2x_1 + 4$; $X_2 = 5x_2 + 30$

The BGN juice was then autoclaved (Beschickung Loading model 100-800, Kempe, Germany) at 121^{o}C for 15 minutes to yield the BGN milk.

3.2.4 Experimental design and data analysis for optimum BGNM

A central composite rotatable design for two independent variables (time and temperature) was performed to determine the optimal flour hydration time and hydration temperature for the optimal BGNM production. The independent variables and their levels are detailed in Table 3.1. The outline of experimental design (12 runs) with the coded levels is given on Table 3.2 following the process described in section 3.2.3; each design point was performed in triplicates, except the centre points that were performed four times. The experiment was carried out in randomised order. Dependent variables were pH, sedimentation, total solids and colour as product quality. The experimental data was fitted to a polynomial regression model for predicting individual responses.

…… Equation 3.1

Where and are intercept, linear, quadratic and interaction regression coefficient terms, respectively, and are independent variables time and temperature, respectively. Statistical significance of the terms in the regression equation was examined by analysis of variance (ANOVA) for each response. Lack of fit and adequate precision was used to judge adequacy of model fit (Design-Expert 8). The fitted model was used to navigate the design space and search for optimum hydration time and temperature for optimum BGNM using numerical optimisation tool in Design-Expert 8.

3.2.5 pH and sedimentation stability determination of BGNM

The pH of the BGNM (20 mL) was measured at room temperature (21 ± 2^{o}C) using a Knick pH-Meter 766 meter, Elscolab, Nederland.

Sedimentation stability of the BGNM was determined using the method of Quasem *et al*. (2009). BGNM (400 mL) was poured into the 400 mL graduation mark of a 500 mL glass cylinders, and covered at the brim using food grade heavy aluminium foil, and kept at refrigeration temperature (4^{o}C) for 24 h. Sedimentation

Table 3.2 Central Composite Rotatable Design (CCRD) arrangement for BGN milk extraction[a]

Test run	Time	Temperature
1	-1	-1
2	-1	+1
3	+1	-1
4	+1	+1
5	0	-1.414
6	0	+1.414
7	-1.414	0
8	+1.414	0
9	0	0
10	0	0
11	0	0
12	0	0

[a]Coded levels of time (-1.414, -1, 0, +1, +1.414) correspond to 1.17. 2, 4, 6, and 6.83 h respectively that of temperature (-1.414, -1, 0, +1, +1.414) correspond to 22.93, 25, 30, 35, 37.07^{o}C respectively.

stability was measured by observing if there was separation into two layers, the lower layer (condensed sediment phase) and the top layer (clear serum phase). The height of the sediments was measured. Stability was expressed as a ratio of the sediment height to the total height.

3.2.6 Total solids

Total solids (TS) of the BGNM were determined using the microwave technique described by Reh & Gerber (2003) with some modification. BGNM (1 g) designated as W_0 was spread onto a 90 mm diameter watch glass, weight of dish and sample was W_1. Heating was in a Samsung microwave with a triple distribution system. The sample and dish was weighed (Mettler AE 163 balance) every two minutes until three consecutive same weights were recorded (W_2). Total solid yield of BGNM was calculated as follows:

——— % ……Equation 3.2

3.2.7 Colour measurements of BGNM

The colour of the BGNM (50 mL) samples was measured using a Hunterlab Colorflex Spectrophotometer 45^o / 0^o standard, set at standard observer 10^o and D65 and standardised with white tile (L* = 93.41, a* = -1.18, b* = 0.75) and black tile (L* = 5.49, a* =-7.08, b* = 4.66). Milk samples were placed in glass samples holder and reflectance measured for L*a*b* and LCh colour scales. L* is lightness with 100 as maximum indicating a perfect reflecting diffuser and the minimum is zero, which is black. The a* and b* axes have no specific numerical limits, negative a* is green, positive is red. Negative b* is blue positive b* is yellow (Anon, 2008 & Aidoo *et al*., 2010). Chroma (C) is the quality that distinguishes a pure hue from a gray shade and describes hue saturation or purity; its axis extends from the values (lightness) axis towards the pure hue (Sahin & Samnu, 2006).

Colour differences between the milk samples were estimated using the colour difference ΔE given as:

Equation 3.3 (Sahin & Samnu, 2006)

where L = lightness, a* = green-red scale, b* = blue-yellow scale.

3.2.8 Validation of BGNM

The hydration of the red BGN flour was done at the estimated optimal time and temperature of 2 h at 25^{o}C, and the pH, hue and total solids determined as discussed earlier in sections 3.2.5, 3.2.6 and 3.2.7.

3.2.9 Production of BGNM from different BGN varieties

Milk was extracted from five BGN varieties (black, brown-red, brown-eye and black-eye) following the method reported earlier in section 3.2.3 and Figure 3.2. However, the flour and water mixture was hydrated (Metter water bath, Lasec, South Africa) in a ratio of 1:10 at 25^{o}C for 2 h as estimated in section 3.2.3.

3.2.10 pH and colour measurements of BGNM

pH and colour of the five BGNM samples were measured as is described previously in section 3.2.5 and 3.2.7 respectively

3.2.11 Antioxidant activity of BGNM

The antioxidant activity of the BGNM was determined using the method described by Peschel *et al*. (2006) and Randhir & Shetty (2007). BGNM (1.5 mL) was mixed with 50% methanol to a concentration µg mL^{-1}, and allowed to react for 10 minutes. The BGNM sample was then mixed with 3.0 mL methanolic 1, 1-diphenyl-2-picrylhy-drazyl (DPPH) solution (20 µg mL^{-1}). The reaction took place in foil covered test tubes and stood for 5 minutes for decolourisation of the solution. Absorbance was measured at 517 nm using a UV/Vis Spectrometer (PerkinElmer Lambda 25). Trolox was mixed with 50% methanol to give µg mL^{-1} and used as the standard.

3.2.12 Sensory analysis of the BGNM

A 39-member panel consisting of staff and students from Cape Peninsula University of Technology were served the four samples (brown, red, black-eye and brown-eye) of the BGNM. Citric acid (0.001%) was added to the milk before serving to the panellist to balance the taste. Each sample (40 mL) was identified by a three-digit random number (Figure 3.3) and was served cold (4-6^{o}C) in a clear atcha tubs, in a well-ventilated and naturally lighted room. Cold tap water was provided to reset

Figure 3.3 Typical bambara groundnut as they were served to the panellists. 459- brown-eye, 624- red, 826- black-eye and 762- brown

the palate. The panellists were required to evaluate each sample and rate their preference based on colour, aroma, taste, consistency and overall acceptability. The rating was on a four point numerical scale labelled from 1- very undesirable, 2- moderately undesirable, 3- moderately desirable and 4- very desirable.

3.2.13 Data analysis and numerical optimisation

All results were reported as mean of three independent trials. Analysis of variance (ANOVA) was used to determine the differences between treatments. Duncan's multiple range tests was used to separate means where differences existed (IBM SPSS, 2010). The optimisation objective was to minimise temperature and pH while maximising hue and total solids BGNM. Design Expert-8 was used to estimate desirability, an objective function that ranges from zero outside of the limits to one at the goal. The numerical optimisation found a point that maximizes the desirability function.

Hierarchical cluster analysis was used to determine the number of clusters inherent in the quality and sensory data. Principal component analysis was used to extract the components that explained the variability in the data. K-mean cluster analysis was used to identify the characteristics of the clusters (IBM SPSS, 2010).

3.3 Results and discussions

3.3.1 Modelling the effect of hydration time and temperature on BGNM

The descriptive statistics for pH, stability, lightness, chroma and total solids are summarised in Table 3.3. The quadratic effect of temperature on pH of BGNM was not significant hence this term was removed from the quadratic model resulting in a reduced quadratic model as shown in Table 3.4. The analysis of variance indicates that the reduced quadratic model (F (0.0042, 0.0003) = 11.61; $p < 0.0001$) was effective in describing the pH of the BGNM. There was only a 0.01% chance that F-value this large could occur due to noise. The model lack of fit ($F(0.002, 0.0003) = 4.48$; $p = 0.0392$) was significant. However the R^2 of 0.4719 was in reasonable agreement with the adjusted R^2 of 0.4312. Furthermore, the adequate precision (25.072) which measures the signal to noise ratio indicates an

Table 3.3 Descriptive statistics for the quality of BGNM as affected by hydration time and temperature

Test run	Time	Temperature	pH	Stability	Lightness (L)	Chroma (C)	Hue(^{o})	Total solids (%)
1	2	25	6.44 ± 0.02	0.51 ± 0.04	50.77 ± 0.51	11.79 ± 0.91	51.91 ± 1.43	2.79 ± 0.24
2	6	25	6.52 ± 0.01	0.23 ± 0.03	50.97 ±0.5250	9.67 ± 0.2950	55.89 ± 2.28	1.29 ± 0.05
3	2	35	6.45 ± 0.01	0.32 ± 0.03	49.7 ±0.11750	11.84 ±0.1300	55.95 ± 2.86	2.64 ± 0.06
4	6	35	6.50 ± 0.01	0.26	51.95 ± 0.81	9.78 ± 1.0600	52.39 ± 6.55	1.94 ± 0.08
5	1.50	30	6.60 ± 0.01	0.19 ± 0.05	49.49 ± 0.38	11.06 ± 0.63	54.73 ± 0.99	1.93 ± 0.18
6	6.50	30	6.52	0.20 ± 0.08	44.52 ±0.45	10.97 ± 0.45	52.19 ± 3.11	1.68 ± 0.01
7	4	22.93	6.52 ± 0.02	0.16 ± 0.09	48.90 ± 0.02	9.77 ± 0.75	62.67 ± 3.17	3.29 ± 0.28
8	4	37.07	6.60	0.20 ± 0.02	45.54 ± 0.27	11.72 ± 0.43	61.06 ± 4.70	1.82 ± 0.12
9	4	30	6.64 ± 0.02	0.24 ± 0.07	48.39 ± 0.82	11.64 ± 0.39	54.83 ± 4.50	1.97 ± 0.28

Table 3.4 Regression coefficients of predicted polynomial model for pH, total solids, hue and lightness of BGNM[a, b]

Coeeficients	Dependent variable			
	pH	Total solids (%)	Hue (angle)	Lightness (L)
Linear				
b_0	6.3404*	3.9339*	26.3452*	54.9095*
b_1	0.0505*	-0.1010*	6.1666*	-1.5852*
b_2	0.0058*	-0.0445*	1.0651	-0.1039
Interaction				
b_{12}	-0.0010*	-	-0.2325	0.0275*
Quadratic				
b_{11}	-0.0035*	-	-	-
b_{22}	-	-	-	-
R^2	0.4719	0.2998	0.1499	0.3406
Adjusted R^2	0.4312	0.2586	0.1150	0.3136
C.V. (%)	0.29	18.82	8.43	3.12
Adeq precision	25.07	9.17	4.75	12.40
Lack of fit	*	*	NS	*

where = time, = temperature.

[b] * = Significant at p = 0.05; NS = Not siginificant at p = 0.05.

adequate signal. A ratio greater than 4 is desirable. Therefore the model was adequate to navigate the design space.

The response surface linear model (Table 3.4) was significant (F(1.21, 0.14) = 7.28; p = 0.0023) in explaining the effect of hydration time and temperature on the total solids of BGNM. There was only a 0.23% chance that F-value this large could occur due to noise. For this model the lack of fit was significant, however, the adequacy precision of 9.168 indicates an adequate signal. Therefore the model was adequate to navigate the design space.

Two factor interaction model (Table 3.4) was significant (F(92.81, 22.22) = 4.29; p = 0.0076) in explaining the effect of time and temperature on the hue of BGNM. There was only a 0.76% chance that F-value this large could occur due to noise. Lack of fit of this model was not significant (F(0.35, 22.22) = 0.016; p = 0.9845). Furthermore, the adequate precision of 4.75 indicates an adequate signal, hence the model was adequate to navigate the design space. Two factor interaction model (Table 3.4) was significant (F(29.28, 1.70) = 12.57; p = <0.0001) in describing the effect of hydration time and temperature on the lightness of BGNM.

3.3.2 Effect of hydration time and temperature on the pH of BGNM

The regression (Table 3.4) for the effect of hydration time (X_1) and temperature (X_2) on the pH of BGNM is given by the equation:

.....Equation 3.4

Time and temperature had a significant ($p \leq 0.05$) effect on the pH. Interaction of time and temperature (F(0.002, 0.0003) = 6.72; p = 0.0123) as well as the quadratic effect of time (F(0.004, 0.0003) = 9.81; p = 0.0029) had a significant ($p \leq 0.05$) effect on the pH of the BGNM. Figure 3.4 is the response surface for the pH of the BGNM as influenced by the hydration time (X_1) and temperature (X_2). The positive coefficient for hydration time indicates increase in the pH of BGNM with time. Similarly, the positive linear coefficient for temperature implies that increase in temperature from 25^{o}C to 35^{o}C resulted in increased pH. Machado *et al*. (1999) also noted that milk pH increases with temperature.

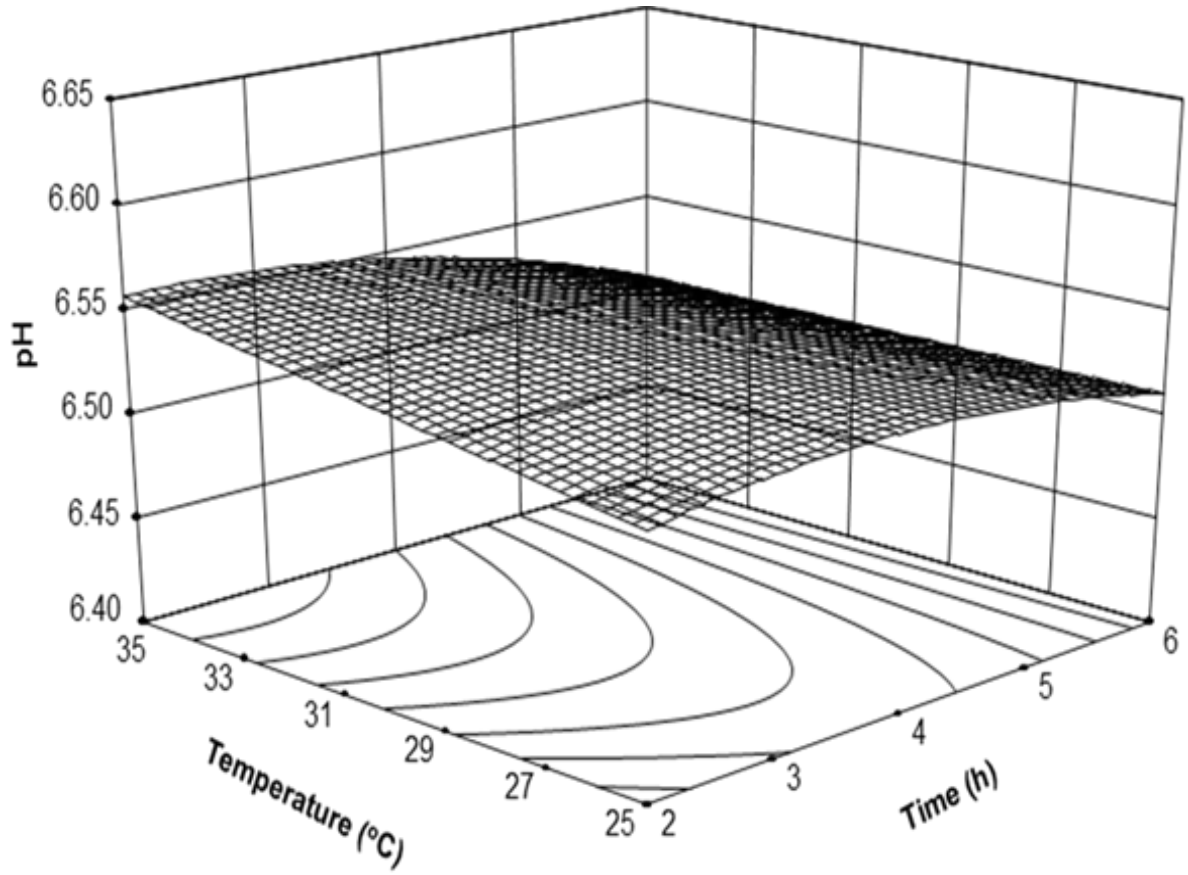

Figure 3.4 Effect of temperature and time on the pH of BGN flour during hydration

The interaction between hydration temperature and time decreased the pH of the BGNM. Increasing both the hydration time and temperature aided in the dissolution of the flour components, which resulted in an increase in pH. The negative quadratic time indicates that the curve is concave meaning that there was a maximum turning point beyond which the pH decreases. The positive linear term for time and the negative quadratic time suggests that the effect of time resulted in increase in pH until a turning point was reached (pH = 6.55; t = 2 h; T = 25^{o}C) beyond which time had a negative impact on pH. The implication was that there was less than linear increase in pH with regards to time because the quadratic term was exerting a downward force on the equation. Perhaps, the longer the hydration, the increase in the activity of microbial flora resulting in fermentation which reduced the pH. This result was in agreement with that of Aidoo *et al*. (2010) who stated that vegetable milk was slightly acidic with a pH range of 6.33-6.97, with Wang *et al*. (2009) who reported vegetable milk to have pH of 6.59 and with Agunbiade *et al*. (2011) who reported a range of 6.1-6.40 for soya milk, BGN milk and yam bean, while that of bovine milk is 6.6 (Yanes *et al*., 2002).

3.3.3 Total solids content of BGNM as affected by hydration time and temperature

The relationship (Table 3.4) between hydration time (X_1) and temperature (X_2) on total solids of BGN flour during hydration could be expressed as:

..... Equation 3.4

The hydration time had a significant effect (F(0.74, 0.14) = 4.45; p = 0.0423) on the total solids of the BGNM. Hydration temperature also had a significant effect (F(1.28, 0.14) = 7.71; p = 0.0089) on the total solids of the milk. Figure 3.5 presents the response surface for the effect of time and temperature on the total solids (%) of bambara groundnut flour during hydration. Increase in time had a negative effect on the total solids, dropping from 2.6 to 1.7%. The temperature had a negative effect on the total solids (%), as the temperature increased from 25^{o}C to 35^{o}C a gradual decrease on the total solids was observed from 1.8% to 1.7%. The decrease in total solids may be as a result of differences in the molecular organisation of the flour.

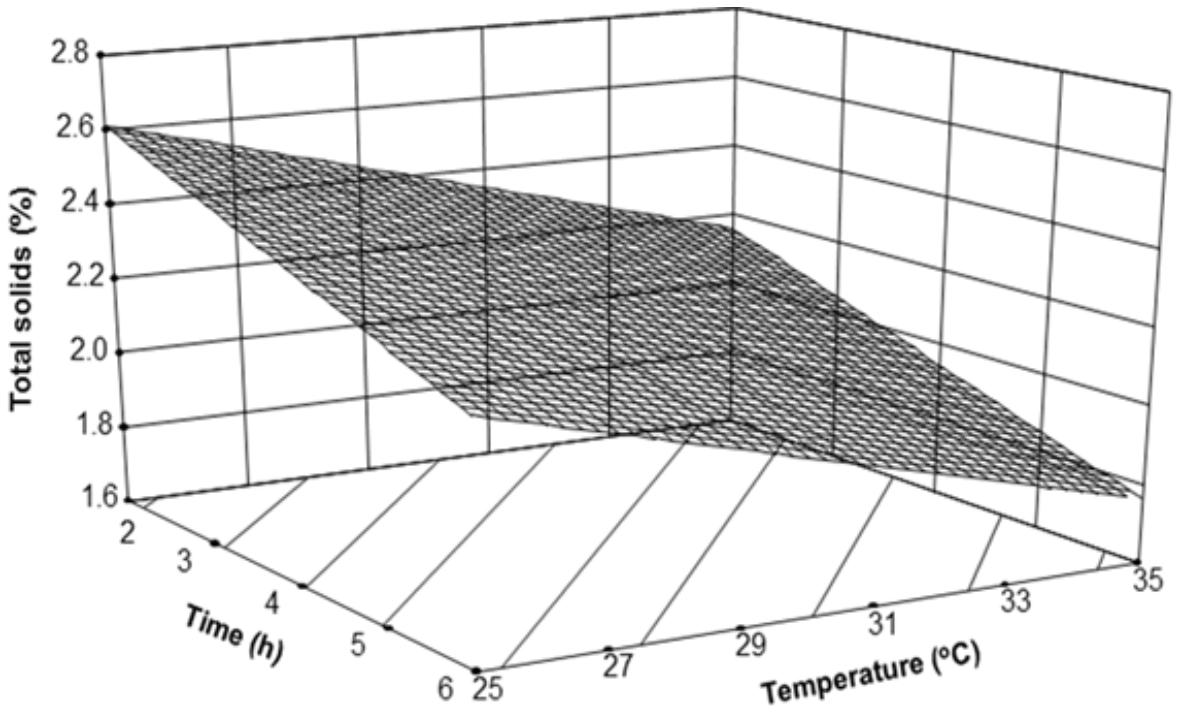

Figure 3.5 Effect of temperature and time on the total solids (%) of bambara flour during hydration

Ukwuru and Ogbodo (2011) reported a range of 20.2-23.2% total solids for tigernut milk. This disparity can be attributed to the sieving step in this study where after hydration, the slurry was filtered through muslin cloth with fine mesh size 707 µm (No. 25) folded four times to yield the BGN juice, hence most of the solid matter was discarded as residue, resulting in a low total solids of the BGNM.

According to Adebowale *et al*. (2002) the effect of temperature on hydration of BGN flour reveals that the flour components hydrate differently due to differences in the molecular organization. With both time and temperature terms negative, the curve showed an accelarating decline in total solids (Figure. 3.5).

Annealing is the treatment of starch in excess of water for an extended period, below the gelatinisation temperature and above the glass transition temperature (Gomes *et al*., 2005). In this experiment it was expected that the starch components in the flour may have undergone annealing since the hydration was in excess of water and the temperature below gelatinisation. Hence, there may have been reorganisation of starch molecules leading to amylopectin double helices acquiring a more organised configuration. The increase in organisation leads to decrease in swelling power and solubilisation of starch (Gomes *et al*., 2005). Furthermore, Jideani and Mpotokwane (2009) reported that increase in temperature resulted in increase in water absorption rate and a decrease in water absorption capacity of BGN seeds. The low solubilisation of the starch may have resulted in the observed decrease in total solids. Machado *et al*. (1999) reported that low concentrated milk solutions have a greater amount of free water, as well as lower viscosity and fat content, which could be true for the BGNM.

3.3.4 Effect of hydration time and temperature on colour of BGNM

Time had a significant effect ($F(83.62, 1.70) = 35.90$; $p = <0.0001$) on the lightness of the BGNM; however, hydration temperature, time and temperature interaction did not affect lightness significantly ($F(0.05, 1.70) = 0.022$; $p = 0.8834$) and ($F(2.43, 1.70) = 1.04$; $p = 0.3109$), respectively. The final equation (Table 3.4) in terms of time (X_1) and temperature (X_2) was:

Equation 3.5

Figure 3.6 depicts the response surface for the effect of temperature and time on the lightness (L) of the BGNM. The interaction of time and temperature

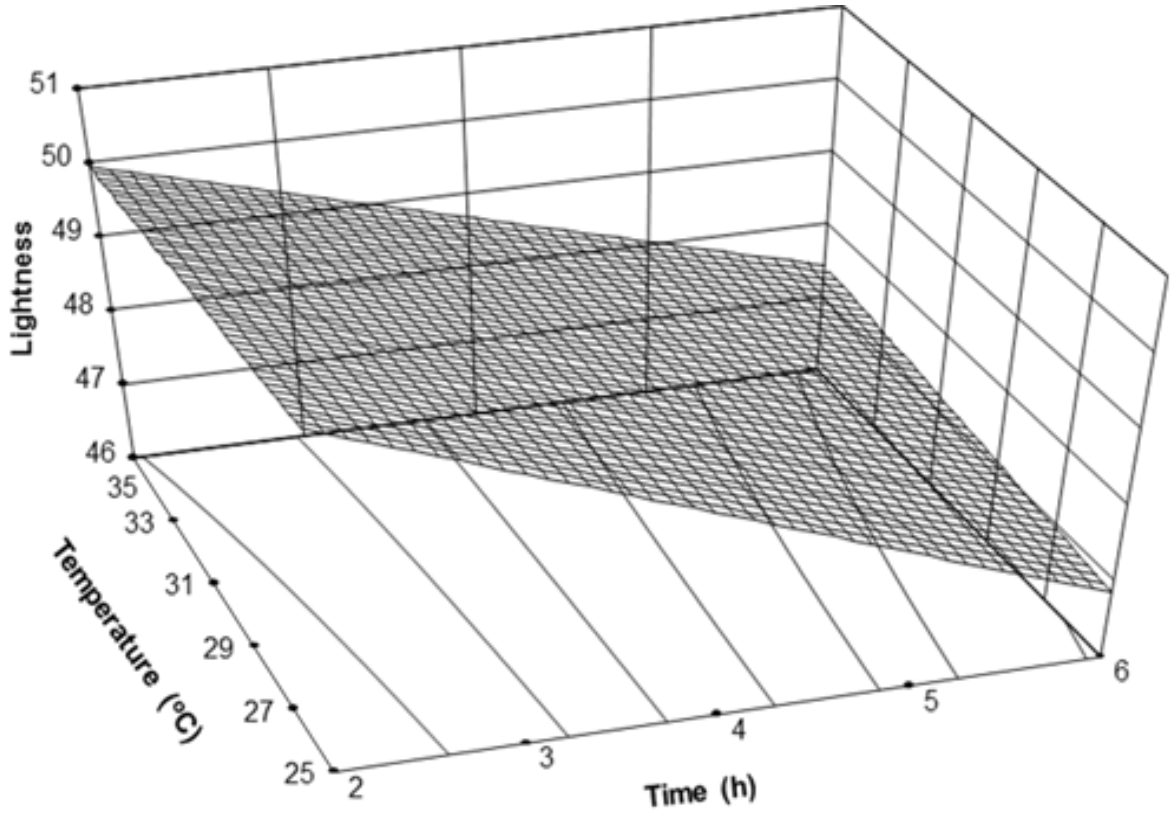

Figure 3.6 Effect of temperature and time on the lightness (L) of bambara flour during hydration

was positive resulting in a slight increase on the lightness of the BGNM. The negative main effects of time and temperature and the positive interaction effect indicated that the effect of time and temperature resulted in accelerated decline in lightness. L is the colour parameter that measures the extent of light, thus L* when 0 would indicate black and when 100 would indicate white. Decrease in L* indicated darkening of the milk (Aidoo, *et al*., 2010). This could have been attributed to the pigment component in the seed coat (Nti, 2009) and enzymatic reactions. The implication is that exposure of BGNM to high temperature could result in darkening of the milk after some time during storage.

The tests of between subjects effects indicated that the hydration time ($F(3.184, 0.35) = 9.12$) as well as temperature ($F(1.803, 0.35) = 5.16$) had significant ($p \leq 0.05$) effect on the chroma. The interaction of time and temperature on chroma was not significant ($p > 0.05$). Table 3.5 indicates the effect of time and temperature on the chroma of BGNM. The effect of time resulted in a decrease in chroma meaning that the BGNM becomes more saturated in hue. This is in agreement with the darkening of the milk. The temperature effect results in an increase in chroma meaning a less saturated hue.

The effect of hydration time on hue of BGNM was significant ($F(94.81, 22.22) = 4.38$; $p = 0.0398$); while hydration temperature did not have any significant effect ($F(23.64, 22.22) = 1.09$; $p = 0.2992$). However, the interaction of time and temperature had a significant effect ($F(178.98, 22.22) = 8.00$; $p = 0.0060$) on the hue. The effect of hydration time (X_1) and temperature (X_2) on BGNM could be described by the relationship (Table 3.4):

– ..... Equation 3.7

The response surface for the effect of hydration time and temperature on the hue of BGNM is detailed in Figure 3.7. Hue of BGNM increased as both the hydration time and temperature increases. However, a negative effect was observed as a result of the interaction between time and temperature indicated by the sharp tilting of the surface in Figure. 3.7.

The positive main effects of time and temperature with the negative interaction suggested that time and temperature exerted an increase in hue. However, the increase is less than linear as the interaction term exerts a downward effect on the graph. Hue is expressed as an angle where 0^o represents

Table 3.5. Effect of time and temperature on the stability, and chroma of BGNM*

Dependent variables	Stability	Chroma
Time		
1.5	0.19 ± 0.05^{a}	11.06 ± 0.63^{a}
2	0.42 ± 0.12^{b}	11.82 ± 0.53^{a}
4	0.21 ± 0.07^{a}	11.19 ± 0.97^{a}
6	0.24 ± 0.02^{a}	9.73 ± 0.64^{b}
6.5	0.20 ± 0.08^{a}	10.97 ± 0.45^{a}
Temperature		
22.93	0.16 ± 0.09^{a}	9.77 ± 0.75^{a}
25	0.37 ± 0.16^{b}	10.73 ± 1.34^{b}
30	0.22 ± 0.06^{c}	11.33 ± 0.51^{b}
35	0.29 ± 0.04^{b}	10.81 ± 1.33^{b}
37.07	0.20 ± 0.02^{c}	11.71 ± 0.43^{b}

*Values are mean ± standard deviation. Values with different superscripts in each column are significantly ($P < 0.05$) different from each other.

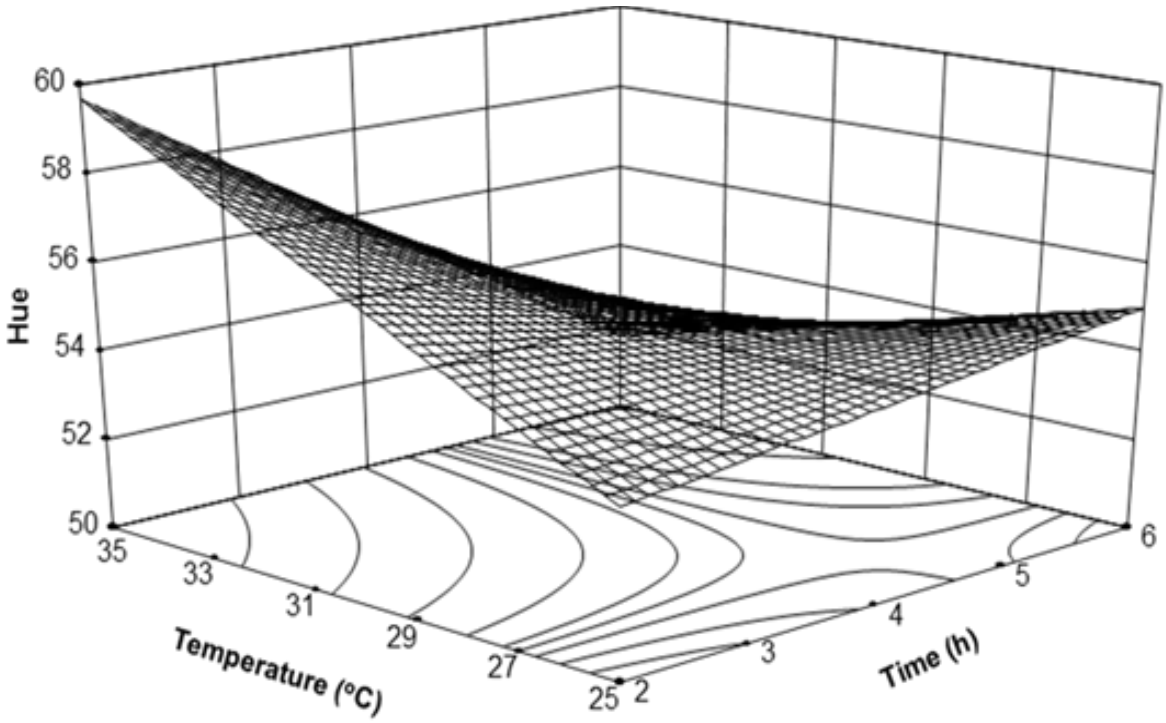

Figure 3.7 Effect of temperature and time on the hue of bambara flour during hydration

red, 90^{o} yellow, 180^{o} green and 270^{o} blue. The hue ranged from 51.91-62.67^{o} indicating a yellowish-red colour. The increases in hue indicated that during hydration more colour components could had solubilised.

3.3.5 Effect of hydration time and temperature on the stability of BGNM

The tests of between subjects effects indicated that the hydration time had significant ($p \leq 0.05$) effect on the stability (F (0.021, 0.003) = 6.31), while the hydration temperature had no significant effect on the stability (F (0.007, 0.003) = 2.19; $p = 0.15$) of the BGNM. The interaction of time and temperature had a significant ($p \leq 0.05$) effect on stability. Table 3.5 indicates the effect of time and temperature on the stability of BGNM. Time and temperature significantly increased the stability of BGNM peaking at 2 h (0.42) and 25^{o}C (0.37) and dropping thereafter.

Time and temperature in a reaction have an influence on the rate and the extent to which the reaction will take place (Clark, 2002). In a system temperature will affect the energy of the particles. Increasing the temperature of the system will comparably increase the amount of kinetic energy the particles have. Thus they move at an increased rate, this impact can result in the reaction reaching an equilibrium or completion (Clark, 2002), which results in a final product with relatively different quality to the reactants (Abu-Ghannam & McKenna, 1997). During the hydration of the BGN flour, time and temperature had an effect on the rate and the type of components which dissolved in the water phase. Increasing time and temperature aided the leaching of minerals and other components from the flour particles into the solution affecting the quality of the BGNM (Kaptso, *et al*., 2007). The hydrating time and temperature was important in the quality of the final product. At a certain time and temperature there was a certain amount and type of flour components which dissolved into the aqueous phase, altering the pH, stability, lightness, chroma, hue and total solids (Kaptso, *et al*., 2007.

3.3.6 Numerical optimisation of time and temperature in BGNM process and process validation

The optimal hydration time and temperature for optimum BGNM was estimated to be 2 h at 25^{o}C, giving desirability of 0.668 (Fig 3.8). The optimal pH, hue and total solids for the optimum BGNM were estimated to be 6.52, 54^{o} and 2.6, respectively.

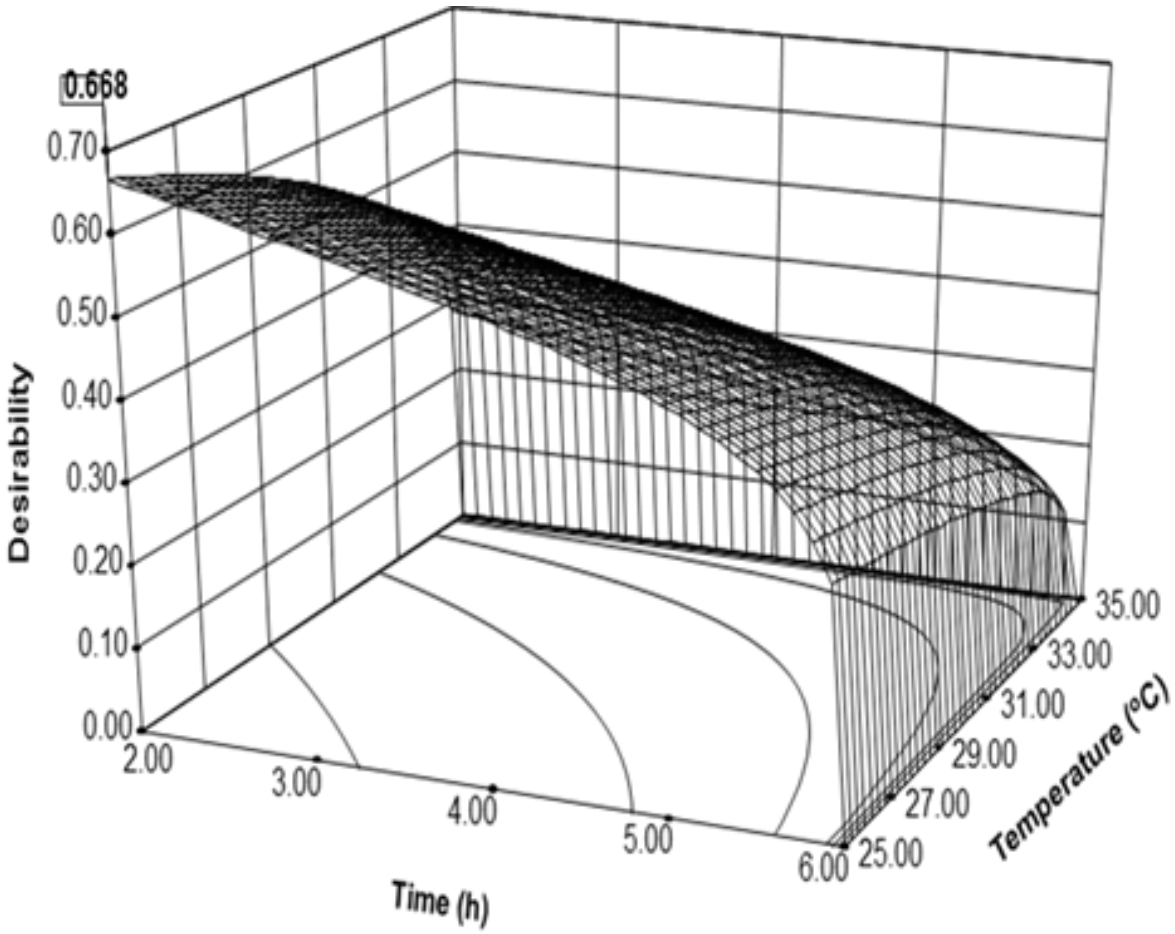

Figure 3.8 Desirability for the minimum temperature and time for minimum pH and maximum hue and total solids

The validated samples were lower in pH (6.42 to 6.46) and higher in total solids (2.89 to 3.25). However, there was no significant difference between the milk in hue (50.36 to 51.36). The difference could be as a result of variation in sieving during the production. Figure 3.9 shows the BGNM which was produced using the determined optimal temperature and time (25^{o}C, 2 h). Therefore, the first objective of optimising the production process of BGNM was achieved.

3.3.7 pH and colour characteristics of BGNM from BGN varieties

The multivariate analysis of variance indicated that BGNM from five different varieties differed significantly in lightness ($F(107.19, 0.142) = 752.6$, $p < 0.05$), chroma ($F(23.143, 0.706) = 32.77$, $p < 0.05$), redness ($F(14.516, 0.789) = 18.41$, $p < 0.05$), yellowness ($F(11.207, 0.1.346) = 8.33$, $p = 0.003$), antioxidant activity ($F(0.0003, 5.982E\text{-}5) = 4.38$, $p = 0.027$) and hue ($F(476.853, 248.511) = 1.92$, $p = 0.184$) while pH ($F(0.002, 0.01) = 1.68$, $p = 0.231$) were not significantly different. Table 3.3 details the descriptive statistics of the BGNM quality. Varietal difference explains the difference in quality. Kapsto *et al*. (2007) and Nti (2009) reported that BGN varieties differ in chemical composition, mineral and tannin content.

The pH of the BGNM ranged from 6.56 for brown to 6.62 for black-eye (Table 3.6). The milk did not differ significantly from each other in pH. The result was in agreement with the report of Aidoo *et al*. (2010), that vegetable milk was slightly acidic with a pH range of 6.33-6.97, and with Wang *et al*. (2009) who reported vegetable milk to have a pH of 6.59. Bovine milk is reported to have a pH of 6.6 (Yanes *et al*., 2002). The pH of the BGNM reported in this work is therefore similar to that of bovine milk.

The lightness (L*) of the milk differed significantly ranging from 41.49 for black variety to 54.34 for brown-eye. L* is the colour parameter that measures the extent of light, thus L* when 0 would indicate black and when 100 would indicate white. Brough *et al*. (1993) reported higher lightness of 58.9 for BGNM. Brough *et al*. (1993) and Nti (2009) produced BGNM from dehulled BGN seeds thereby removing the pigments on the seed coat resulting in lighter milk. Whereas flour from whole milled BGN was used in the present study. The highly pigmented seed coats (black, brown, red) produced BGNM with comparatively darker colour. The difference in seed colour of BGN contributed to the difference observed in lightness between the milk samples, this is supported by Nti (2009) who reported

Figure 3.9 BGNM processed at 25^{o}C for 2 h optimal temperature and time.
BGNM – Bambara groundnut milk

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Table 3.6 Colour, antioxidant and pH characteristics of BGNM from five varieties*

	Black	Brown	Brown-eye	Black-eye	Red
pH	6.60 ± 0.01	6.56 ±0.06	6.62 ± 0.06	6.60 ± 0.01	6.56 ± 0.06
Lightness	41.49 ± 0.27^{a}	44.42 ± 0.42^{b}	53.00 ± 0.16^{d}	54.34 ± 0.41^{c}	42.95 ± 0.52^{e}
Chroma	9.21 ± 1.07^{a}	7.76 ± 0.28^{b}	4.12 ± 1.17^{c}	3.86 ± 0.66^{c}	9.70 ± 0.70^{a}
Hue	54.07 ± 5.64^{a}	33.20 ± 24.63^{b}	63.40 ± 15.62^{a}	64.53 ± 15.53^{a}	51.60 ± 10.92^{a}
Redness	5.42 ± 1.24^{a}	5.04 ± 0.28^{a}	1.39 ± 0.68^{b}	1.55 ± 0.74^{b}	5.89 ± 1.14^{a}
Yellowness	7.41 ± 0.64^{a}	5.89 ± 0.51^{a}	3.56 ± 1.12^{b}	3.70 ± 0.45^{b}	7.55 ± 1.64^{a}
Antioxidant	0.3925 ± 0.0039^{a}	0.3950 ± 0.0005^{b}	0.4135 ± 0.0022^{c}	0.40761 ± 0.0001^{c}	0.4098 ± 0.0167^{c}

*Values are mean ± standard deviation. Values with different superscripts in the same row are significantly different ($p < 0.05$). BGNM - Bambara groundnut milk

darkening of milk to be associated with the pigment component in the seed coat. The lower lightness values could also be attributed to enzymatic chemical reactions during hydration of BGN flour.

The BGNM in terms of chroma ranged from 3.86 (brown-eye) to 9.70 (red). The milk samples differed significantly ($p < 0.05$) in chroma. Milk from black (9.21) and red (9.70) varieties were significantly higher in chroma than the others. The brown (7.76) variety was significantly different in chroma compared to others. There was no significant difference between the brown-eye and the black-eye milk, both being less saturated than the others. The two varieties both had cream coloured seed coats, being less saturated in hue.

In terms of hue the milk ranged from 33.20^{o} (brown) to 64.53^{o} (black-eye). Milk sample from brown BGN was significantly ($p < 0.05$) lower in hue compared to others. However, no difference exists in hue between black, brown-eye, black-eye and red BGNM, their values ranged from 51.06 to 64.53 indicating yellowish-red colour.

The a* on the colour scales defines green (negative a*) to red (positive a*). The positive a* values (1.39 to 5.89) of the milk indicate the redness of the BGNM. Milk from brown-eye and black-eye seeds is significantly lower in redness compared to the others. There was no significant difference in redness between black, brown and red BGNM. Negative b* defines blueness while positive b* defines yellowness in a sample. The positive values indicate the yellowness of the samples ranging from 3.56 (brown-eye) to 7.55 (red). The brown-eye and the black-eye were significantly lower in yellowness compared to the others. No difference exists between the milk from black, brown and red in yellowness. The brown-eye and the black-eye milk were characterised by low redness, yellowness and high hue but less saturated. Milk from black, brown and red were characterised by comparatively higher redness, yellowness and lower hue with high saturation. The variable colours of the milk will be of an advantage in segmenting the market for the commercialisation of the milk.

Lightness correlates negatively with chroma ($r = -0.925$, $p < 0.01$), redness ($r = -0.898$, $p < 0.01$) and yellowness ($r = -0.835$, $p < 0.01$). BGNM with high lightness (brown-eye, black-eye) was low in chroma, redness and yellowness. There was a positive correlation between chroma and redness ($r = 0.889$, $p < 0.01$), negative correlation with pH ($r = -0.448$, p 0.029). Hue negatively correlates

with redness (r = -0.598, p = 0.005). Redness was positively correlated with yellowness (r = 0.703, p = 0.001). Yellowness correlated negatively with pH (r = -0.530, p = 0.016).

3.3.8 Colour differences between the BGNM samples

Colour difference (∆E) of 1 is defined as a just-noticeable difference (JND), the threshold at which a trained observer would notice a difference between two colours. There is the concept of perceived and acceptable colour difference with JND. In colour imaging ∆E of 4 to 8 is deemed acceptable (Sharma, 2004). The colour differences between the samples ranged from 1.4 to 13.9 (Figure 3.10). The differences will be therefore perceivable by the consumers since the values are greater than 1. The colour differences between black versus brown and red, brown versus red, and brown-eye versus black-eye may be acceptable since they are within less than 4. However, the differences between black versus brown-eye, and black-eye; brown versus brown-eye and black-eye; brown-eye versus red and black-eye versus red may not be acceptable as they were greater than 8. The objective of optimising the BGNM production was achieved.

3.3.9 Antioxidant activity of BGNM from different varieties

The antioxidative capacity (Table 3.5) of the BGNM ranged from 0.3925 to 0.4135 A and differed significantly ($p < 0.05$) from each other. Similar result was reported by Nti (2009), the antioxidant activity among BGN varieties differs significantly, which could be attributed to the varietal difference. The hydrogen or electron donating capability of the extracts led to the reduction of the DPPH (Berk *et al*., 2011). During the reduction reaction there is discolouration of the test sample from a bright purple to a clear solution, thus most of the UV is transmitted through the glass cuvet other than being absorbed by the DPPH, hence the lower the A the higher the antioxidative capacity. In the present study Trolox, the standard, had the least absorbance (0.3234 A), this was because it had reduced the most of the DPPH. The same concentrations of BGNM and Trolox were used, thus the reading were compared. The black had 0.3925 A close to that of the Trolox (0.3234 A), it had the most antioxidative capacity, while brown, black eye, red and brown eye were 0.3950 A, 0.4076 A, 0.4098 A and 0.4135 A respectively. These were the anticipated outcome since the pigment component is located in the seed coat

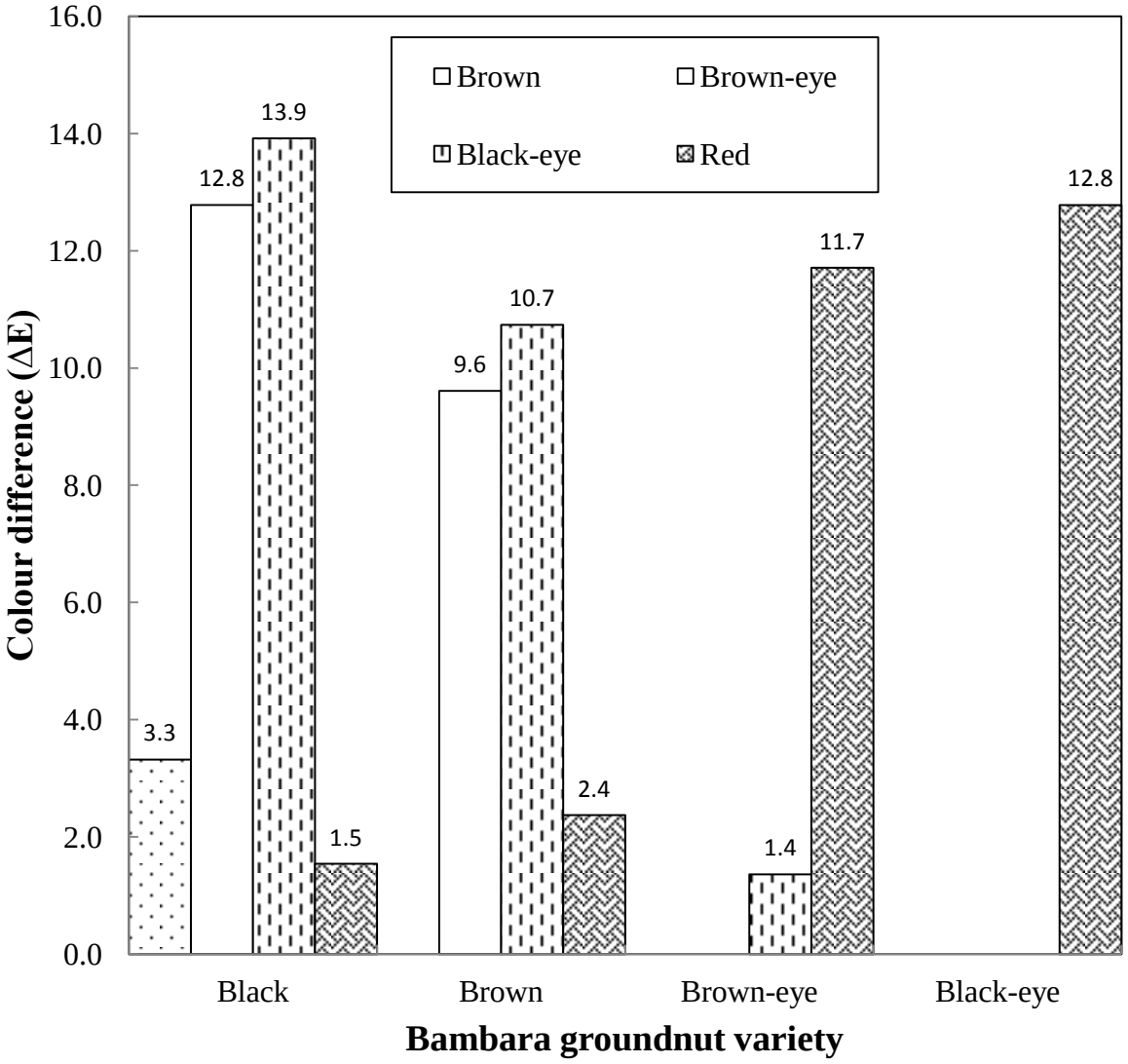


Figure 3.10 Colour differences between the bambara groundnut milk

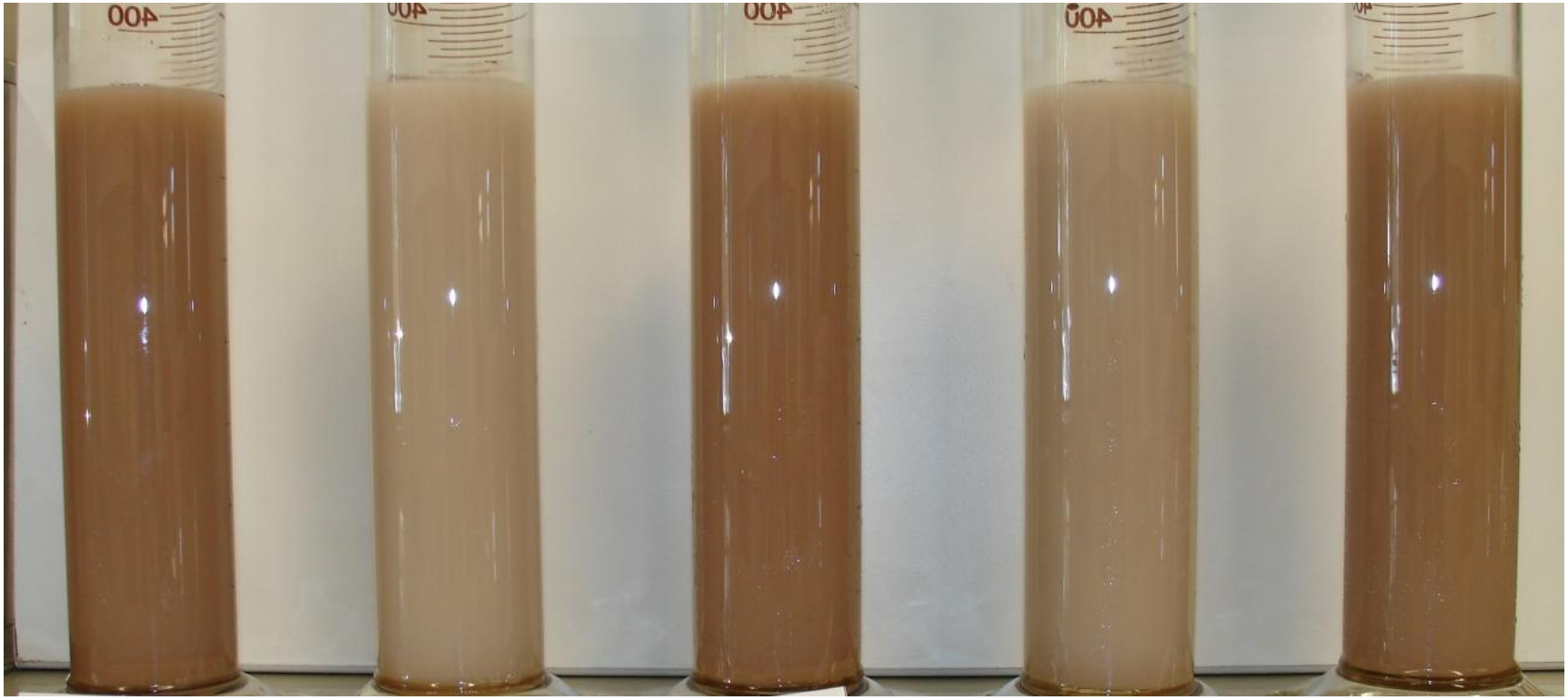
(Tsuda *et al*., 1994) and it's the pigment component which has the highest antioxidative ability (Nti, 2009). Figure 3.11 shows the five BGNM extracted from red, brown, black, brown-eye and black-eye BGN. Producing BGNM milk in this way from whole meal BGN flour will provide nutritional benefit due to its antioxidative activity.

3.3.10 Clustering the BGNM on the basis of colour, pH and antioxidant activity

The dendrogram (not shown) obtained from the hierarchical cluster analysis indicated that the BGN milk could be divided into two groups on the basis of colour, pH and antioxidant activity. The colour parameters, antioxidant activity and pH of the milk samples from different varieties of BGN were subjected to principal component analysis (PCA) to identify the two variables that form the two clusters. The suitability of the data for factor analysis was assessed prior to performing PCA. Inspection of the correlation matrix revealed the presence of many coefficients of 0.3 and above. The Kaiser-Meyer-Oklin value (0.610) exceeded the recommended value of 0.6. The Barlett's test of sphericity was significant ($p < 0.05$). Hence, factorability of the correlation matrix was supported.

PCA revealed that the variation in the characteristics of the BGNM from different varieties could be explained by two components with eigenvalues exceeding 1. Much of the variation (44.5%) in the data was explained by component 1 and 34.9% by component 2, with a cumulative variation of 79.4% of the variation explained by the components. This was further supported by the clear break after the second component in the scree plot (not shown).

Varimax rotation was performed to aid in the interpretation of these two components (Table 3.7). Yellowness loaded strongly on component 1, while hue loaded strongly on component 2. The implication was that BGNM can be categorised into two groups on the basis of yellowness and hue. K-means cluster analysis was performed to characterise the two milk groups on the basis of these quality parameters. Cluster 1 was the milk samples with high hue (74.7^{o}, yellowish-red) but comparatively less in yellowness ($b = 5.19$). Cluster 2 was those with less hue (48.4^{o}, reddish-yellow) and comparatively high in yellowness ($b = 5.44$). Table 3.8 indicates the cross-tabulation of the milk samples according



Red **Brown eye** **Black** **Black eye** **Brown**

Figure 3.11 Bambara groundnut milk extracted from the five varieties of bambara groundnuts

Table 3.7 Pattern/structure for coefficients obtained using varimax rotation of two factor solution for bambara milk from different varieties

	Component	
Quality	1	2
Yellowness	0.961	-0.171
Chroma	0.880	-0.428
Lightness	-0.735	0.636
pH	-0.677	0.042
Hue	0.047	0.934
Redness	0.586	-0.772
Antioxidant	-0.268	0.597
% of variance explained	44.5	34.9

to their cluster membership. Milk from black, brown and red belong to cluster 2 with less hue and high in yellowness.

3.3.11 Sensory characteristics of BGNM from different BGN varieties

The demography of the panellists whom are yoghurt consumers is indicated in Table 3.9. There were 39 panellists 87.2% of whom were females, 92.3% were international students and 5.1% were less than 20 years old, 89.7% within 20-30 years of age and 5.1% within 31-40 years of age.

Multivariate analysis of variance indicated that the panellist differed significantly ($p < 0.05$) in their rating for appearance, colour, aroma, taste, mouth feel and overall acceptability. The population consisted of people from different walks of life. The difference in rating amongst the panellist was expected, since people from different age groups (>20-40 yrs), and occupation (students, lecturers and non-academic staff) participated in the study. The four BGNM samples were significantly different in terms of appearance ($F (2.778, 0.51) = 5.444$, $p = 0.002$), colour ($F (4.530, 0.499) = 9.074$, $p < 0.05$), mouthfeel ($F (1.374, 0.431) = 3.188$, $p = 0.026$) and overall acceptability ($F (2.137, 0.540) = 3.955$, $p = 0.01$), while the aroma ($F (0.297, 0.433) = 0.686$, $p = 0.562$) and taste ($F (0.536, 0.431) = 1.244$, $p = 0.297$) were judged to be the same. The differences in appearance, colour, mouthfeel and overall acceptability may be attributed to the varietal differences.

The panellists mean rating for appearance ranged from 2.2 for black-eye and brown-eye to 2.7 for red (Table 3.10), in specific terms, from moderately undesirable to moderately desirable. BGNM from the red variety was rated significantly ($p < 0.05$) higher in appearance compared to others. Appearance which is the opacity of milk is due to its content of suspended particles of protein and certain minerals. According to Nti (2009), BGN varieties differ in their carbohydrates content. Starch gelatinisation during the sterilisation step resulted in the rich appearance of the milk. The differences in chemical composition and pigment components also impacted the panellist rating for appearance. In terms of colour the panellists mean rating (Table 3.10) ranged from 2.1 (moderately undesirable) for brown-eye to 2.9 (moderately desirable) for red.

The BGNM from the red variety was rated significantly ($p < 0.05$) higher in colour, followed by brown, with significantly low rating for brown-eye and black-eye

Table 3.8 BGNM from different varieties classified into their clusters based on pH, colour and antioxidant activity*

Bambara groundnut milk	Cluster Number of Case**		
	1	2	Total
Black	0	4 (100)	4 (100)
Brown	0	4 (100)	4 (100)
Black-eye	2 (50)	2 (50)	4 (100)
Brown-eye	2 (50)	2 (50)	4 (100)
Red	1 (25)	3 (75)	4 (100)
Total	5 (25)	15 (75)	20 (100)

*Numbers are frequency and percentage in bracket.

**Cluster 1 = high hue (74.7^{o}, reddish-yellow) but less in yellowness (b = 5.19); Cluster 2 = less hue (48.4^{o}, reddish-yellow) and high in yellowness (b = 5.44).

Table 3.9 Demography of the panellists*

Item	Frequency (Percentage)
Gender	
Male	5 (12.8)
Female	34 (87.2)
International student?	
No	3 (7.7)
Yes	36 (92.3)
Age category	
<20	2 (5.1)
20-30	35 (89.7)
31-40	2 (5.1)

*Numbers are frequency and percentage in bracket.

Table 3.10 Effect of variety on the sensory parameters of BGNM*

Dependent variables	Red	Brown-eye	Brown	Black-eye
Appearance	2.7 ± 0.8[a]	2.2 ± 0.8[b]	2.3 ± 0.8[b]	2.2 ± 0.9[b]
Colour	2.9 ± 0.8[a]	2.1 ± 0.9[b]	2.5 ± 0.9[c]	2.2 ± 0.9[b]
Aroma	2.0 ± 0.9[a]	2.0 ± 0.9[a]	2.0 ± 0.8[a]	2.2 ± 0.8[a]
Taste	1.9 ± 0.9[a]	1.9 ± 0.9[a]	1.9 ± 0.9[a]	2.1 ± 0.9[a]
Mouthfeel	2.2 ± 1.0[a]	1.9 ± 0.9[b]	2.0 ± 1.0[a]	2.2 ± 0.9[a]
Overall acceptability	2.5 ± 0.9[a]	2.0 ± 0.8[b]	2.2 ± 1.0[a]	2.3 ± 0.9[a]

*Values are mean ± standard deviation. Values with different superscripts in each row are significantly ($p < 0.05$) different from each other.

The red BGNM had a pronounced chocolate brown colour, while the brown seed milk was pale brown; the cream coated seeds (brown-eye and black-eye) had dirty-white colour. The pigment components which are located in the seed coat easily leached into the aqueous phase during the hydration step impacting the characteristic testa colour of each variety to the milk.

The panellists could not detect any difference in the samples in aroma and taste both being moderately undesirable. The low rating for aroma and taste was expected as the samples were served without sugar and being a legume, the consumers need to acquire the unique taste of legume milk.

Mean rating for mouthfeel ranged from 1.9 (brown-eye) to 2.2 for both red and black-eye. Mouthfeel is the BGNM physical and chemical interaction with the mouth. The difference perceived in mouthfeel was as a result of the varietal differences. Tannins, which are located in the seed coat, also contributed to the mouthfeel. The dark coloured seed coats have the highest amount of tannins, which when ingested give a certain sensation in the mouth. This was mostly pronounced in the red and brown varieties, and less in the brown-eye and black-eye varieties. However, it was expected that the high temperature and pressure of sterilisation along with the initial hydration of the flour would have reduced the antinutritional factors in the seeds.

Overall acceptability of the samples ranged from 2.0 (brown-eye) to 2.5 (red). The milk from brown-eye seeds was significantly lower in overall acceptability being moderately undesirable. There was no significant difference in overall acceptability among the BGNM from red, brown and black-eye, however, the red BGNM was moderately desirable with a mean rating of approximately 3. Red BGNM was the most preferred both in appearance, colour and overall acceptability since it resembled chocolate milk familiar to most of the panellists. However in the study by Brough *et al*. (1993) the white coloured milk was most preferred to the other darker colour. In this study the BGNM from cream (brown-eye, black-eye) varieties had a dirty-white appearance which is not expected of normal bovine milk, this may explain the down rating of the samples. However, the BGNM from red variety was most preferred. The objective of producing consumer acceptable BGNM was achieved, while the hypothesis which stated that the BGNM would be accepted by the consumers was tested and accepted.

3.3.12 Market segmentation of BGNM based on consumer preference

The dendrogram (not shown) obtained from the hierarchical cluster analysis indicated that the panellists could be divided into two groups on the basis of milk appearance, colour, aroma, taste, mouthfeel and overall acceptability. The sensory quality indices (appearance, colour, aroma, taste, mouthfeel and overall acceptability) were subjected to principal component analysis (PCA) to identify the two variables for the grouping. The suitability of the data for factor analysis was assessed prior to performing PCA. Inspection of the correlation matrix revealed the presence of many coefficients of 0.3 and above. The Kaiser-Meyer-Oklin value (0.812) exceeded the recommended value of 0.6. The Barlett's test of sphericity was significant ($p < 0.05$). Hence, factorability of the correlation matrix was supported.

PCA revealed that the variation in the sensory characteristics of the BGNM from different varieties could be explained by two components with eigenvalues exceeding 1. Much of the variation (44.9%) in the data is explained by component 1 and 31.4% by component 2, with a cumulative variation of 76.3% of the variation explained by the components. This is further supported by the clear break after the second component in the scree plot (not shown).

Varimax rotation was performed to aid in the interpretation of these two components (Table 3.11). Taste and mouthfeel loaded strongly on component 1, while appearance and colour on component 2. The component score coefficient matrix indicated that component 1 was taste while component 2 correlated to appearance and colour. The implication is that BGNM can be categorised into market groups on the basis of taste, appearance and colour. K-means cluster analysis was performed to characterise the groupings on the basis of these sensory attributes. Cluster 1 was the BGNM that are moderately undesirable (2) in taste and moderately desirable (3) both in appearance and colour. Cluster 2 was those that are moderately undesirable (2) both in taste, appearance and colour. Hence, taste, appearance, and colour were the important parameters considered by the panellists in their preference. Brough *et al*. (1993) reported taste, colour and viscosity as important to the panellists in their study. Hence, in future work attention will be given to taste and appearance using the preferred variety (red).

Table 3.11 Pattern/structure for coefficients obtained using varimax rotation of two factor solution for sensory attributes of bambara milk from different varieties

	Component	
Quality	1	2
Taste	0.906	0.099
Mouthfeel	0.879	0.207
Overall acceptability	0.736	0.387
Aroma	0.669	0.310
Appearance	0.215	0.895
Colour	0.250	0.885
% of variance explained	44.9	31.4

Table 3.12 indicates the cross-tabulation of the milk samples according to their cluster membership with respect to age and BGN variety. In terms of variety, the BGNM from red (74.4%) and the brown (53.8%) belong to group 1- moderately undesirable (2) in taste and moderately desirable (3) both in appearance and colour. Cluster 2 consisted of brown-eye (61.5%) and black-eye (53.8%) being moderately undesirable (2) both in taste, appearance and colour. The entire panellist less than 20 years old, 20-30 years (48.6%) and majority in age 31-40 (87.5%) equivalent of 53.2% of the cases were in group 1, indicating they moderately desire the BGNM milk in appearance and colour but the taste is moderately undesirable. There is high preference for BGNM across all the age groups. The high preference for panellist <20 signals the high probability of acceptance of BGNM if commercialised. Future work will pay attention to masking the slight beany flavour observed by some of the panellists thereby improving the taste. The objective of investigating the effect of variety on the quality of BGNM in terms of colour, pH, antioxidant and consumer acceptability was achieved. The hypothesis that the consumers will accept BGNM was accepted.

3.4 Conclusion

Optimal BGNM could be produced from BGN flour by hydrating the flour at 25^{o}C for 2 h. The pH, hue and total solids of the milk were estimated to be 6.52, 54^{o} and 2.6, respectively. The possibility of producing BGNM from hydrated flour is beneficial as (1) there is huge saving on energy; (2) the milk will contain all the goodness in the seeds especially the pigments on hulls which includes phenolics and flavonoids which may contribute to nutritional well-being.

Quality of BGNM was affected by variety in terms of pH, colour and antioxidant activity. BGNM could be classified into two groups on this basis. Cluster 1 is the milk samples with high hue (74.7^{o}, reddish-yellow) but less in yellowness (b = 5.19). Cluster 2 is those with less hue (48.4^{o}, reddish-yellow) andhigh in yellowness (b = 5.44). Milk from black, brown and red belong to cluster 2 with less hue and high in yellowness, while black-eye and brown-eye belong to cluster 1 having high hue but less in yellowness. Variety also affects consumer

Table 3.12 BGNM from different varieties clusters on the basis of sensory attributes*

Sample	Cluster Number of Case**		Total
	1	2	
Bambara groundnut milk			
Brown	21 (53.8)	18 (46.2)	39 (100)
Black-eye	18 (46.2)	21 (53.8)	39 (100)
Brown-eye	15 (38.5)	24 (61.5)	39 (100)
Red	29 (74.4)	3 (75)	4 (100)
Total	83 (53.2)	73(46.8)	156 (100)
Panellists age			
<20	8 (100)	0	8 (100)
20-30	68 (48.6)	72 (51.4)	140 (100)
31-40	7 (87.5)	1 (12.5)	8 (100)
Total	83 (53.2)	73 (46.8)	156 (100)

*Numbers are frequency and percentage in bracket.

**Cluster 1 = moderately undesirable (2) in taste and moderately desirable (3) both in appearance and colour; Cluster 2 = moderately undesirable (2) both in taste, appearance and colour.

acceptability of BGNM. Taste, appearance, and colour were the important parameters considered by the panellist in their preference. In terms of variety, the BGNM from red (74.4%) and the brown (53.8%) were moderately undesirable (2) in taste and moderately desirable (3) both in appearance and colour. Preference for BGNM was high among all the age groups, demonstrating the potential of BGNM when commercialised.

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CHAPTER 4

OPTIMISATION OF LACTIC ACID BACTERIA GROWTH IN BAMBARA GROUNDNUT MILK AND THE PRODUCTION OF BAMBARA GROUNDNUT PROBIOTIC BEVERAGE AND ITS PHYSICAL AND SENSORY CHARACTERISTICS

Abstract

Bambara groundnut milk (BGNM) from red BGN was inoculated with lactic acid bacteria (LAB) following a three-factor factorial design (4 × 3 × 3) consisting of probiotics (*Lactobacilus acidophilus, L. bulgaricus, L. casei* and *L. plantarum*), temperature (35, 40, 45^{o}C) and fermentation time (6, 15, 24 h). The dependent variables of interest (pH, probiotic growth (OD), lactic acid) were modelled using quadratic polynomial and numerical optimisation was used to estimate the optimal conditions for the production of BGN probiotic beverage (BGNPB). The quadratic polynomial model was adequate to navigate the design space. The LAB and incubation time had a significant effect on the pH, lactic acid and LAB growth (OD) of the BGNM. *L. bulgaricus* significantly ($p < 0.05$) reduced the pH of BGNM to 5.18. There was no significant effect of temperature on pH. Fermentation for 24 h significantly ($p < 0.05$) reduced the pH to 5.14, increased the viability of the bacteria with high absorbance of 0.1816 and increased the lactic acid production to 1.46%. There was no difference in lactic acid production between *L. acidophilus* and *L. plantarum*. The optimal condition for the production of BGNPB was estimated to be *L. bulgaricus* fermented at 35^{o}C for 24 h with a desirability of 0.854. This condition is estimated to yield a pH of 4.7 with optical density of 0.25 at 600 nm. The next promising probiotic was *L. plantarum* at 35^{o}C for 24 h with estimated pH of 4.4 and optical density of 0.20 at 600 nm with 0.843 desirability. Thus it has been established

that BGNM can support the growth of LAB a requirement for production of probiotic beverage. BGN probiotic beverage (BGNPB) was produced by inoculating the red BGNM with *L. bulgaricus* and *L. bulgaricus & L. plantarum* (in combination), fermenting at 35^{o}C for 24 h. Four batches were produced namely (1) *L. bulgaricus* plain; (2) *L bulgaricus & L. plantarum* (in combination) plain; (3) *L. bulgaricus* sweetened and flavoured; and (4) and *L. bulgaricus & L. plantarum* (in combination) sweetened and flavoured. Beverages 1 and 2 were investigated for their pH, susceptibility to syneresis, water holding capacity and viscosity, while all four samples were subjected to sensory evaluation using 40 consumer panellists. The BGNPB physical properties were 4.39 ± 0.01 and 4.29 ± 0.01 for pH, 33.99 ± 3.06 and 38.53 ± 0.61 for syneresis, 62.75 ± 1.52 and 62.33 ± 0.29 for water holding capacity, and 62.00 ± 1.00 and 98.00 ± 1.00 for viscosity for sample 1 and 2 respectively. The four BGNPB samples were significantly different ($p < 0.05$) in aroma, taste, mouthfeel and overall acceptability but not in appearance and colour. BGNPB could be classified into two groups based on appearance, colour, taste and aroma. *L. bulgaricus* sweetened BGNPB was moderately desirable in appearance and colour. Majority of the panellist between 31-40 (62.9%) and majority in age > 40 (58.3%) equivalent to 44.2% of the panellists were group 1, indicating they moderately desired the BGNPB in mouthfeel and taste but the appearance and colour were taste were moderately undesirable. There was high preference for BGNPB across all the age groups. The high preference for panellist >31 indicated the high probability of acceptance of BGNPB if commercialised. Future work will pay attention to increase the sucrose thereby improving the taste.

4.1 Introduction

Milk, juice, vegetable extracts, and cereals fermented with lactic acid are functional foods, better known as probiotic carriers (Granato *et al*., 2010). The lactic acid bacteria (LAB) has technical benefits on the production of the beverage (Shimakwa *et al.*, 2003; Schillinger *et al*., 2005); they bring about the fermentation process, influence the taste, flavour, body and texture and the shelf life and keeping qualities of the final product (Kamaly, 1997). LAB fermented beverage and foods also play a

vital role in people's diets (Oliveria *et al.,* 2002; Shimakawa *et al*., 2003 & Furet *et al.,* 2004). Probiotics are live microbial feed supplements which beneficially affect the host by improving its microbial balance (Salminen *et al.,* 1999). When consumed in adequate numbers they confer a health benefits on the host (Kamaly, 1997; Shimakwa *et al.*, 2003; Schillinger *et al*., 2005; Champagne & Gardener, 2008). The genera *Lactobacillus* and *Bifidobacterium* are normally used as probiotics (Champagne *et al.,* 2009). However, they are selected on their ability to grow and survive in the required media.

Yoghurt production is done world-wide as controlled co-cultured fermentation of milk with two species of lactic acid bacteria namely *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp*. bulgaricus* (Tamime & Robinson, 1999; Oliveria *et al.*, 2002). LAB are mainly used in dairy industry as probiotic microorganisms (Kneifel *et al*, 1993). Lactic acid beverage containing probiotic bacteria is an interesting way to diversify these products, giving them a health promoting function (Oliveira *et al*., 2002). Probiotic bacteria could be added to lactic acid beverage during the final mixing of the product; however the best approach will be to use the probiotics as the starter cultures (Oliveira *et al*., 2002). Probiotics tend to grow slowly in milk because of lack of proteolytic activity; the usual practice is to add the conventional yoghurt bacteria to enhance the fermentation. However, *L. delbrueckii* ssp. *bulgaricus* is argued to cause post acidification which may affect the viability of probiotics (Oliveira *et al*., 2002).

Vegetable milk being different from the dairy milk is treated in such a way to make it more favourable to yield a product similar to their dairy counterparts (Granato *et al.,* 2010). The technological advances have made possible the alteration of some structural characteristics of fruit and vegetables matrices making them ideal substrates for probiotic cultures (Granato *et al.,* 2010). Such modifications include among others pH and fortification of culture media. Many studies have reported that soybean (*Glycine max*) products fermented with lactic acid bacteria (LAB) can provide unique probiotic foods for human nutrition (Kneifel *et al.,* 1993; Heller, 2001; Oliveria *et al*., 2001; Shimakawa *et al.,* 2003; Granato *et al.,* 2010). In Chapter two we had reported the production of consumer acceptable bambara groundnut milk (BGNM)

from red and brown varieties with high antioxidative activity. Numerous studies have been done on BGNs, however, nothing is known about bambara milk as a probiotic carrier (Igbedioh *et al.,* 1994; Alobo, 1999; Amadou *et al.,* 2001; Adebowale *et al.,* 2002; Jideani & Mpotokwane, 2009; Alakali *et al.,* 2010). BGNM contains beneficial nutrients, such as minerals, amino acids (Uvere *et al,* 1999), vitamins and bioactives (Nti, 2009). Like soybean yoghurt, lactic acid fermented BGNM may be suitable probiotic bacterial strain carrier to the host because of its inherent presence of antioxidants, chemical and nutritional composition of BGN. Our objectives were to: (1) investigate the survival of LAB in BGNM with a view to identify the best strain for BGN probiotic (BGNPB); (2) produce BGNPB with a view to developing value-added product and (3) establish the quality and consumer acceptability of BGNPB.

4.2 Materials and methods

4.2.1 Source of BGN, starter cultures and chemicals

Bambara groundnuts (BGN) were purchased from Thusano Products, Louis Trichardt, Limpopo, South Africa. The BGN seeds were sorted into varieties, according to testa and hilum colours; black, red, brown, black-eye and brown-eye. Only the red variety which produced the consumer acceptable BGNM from the previous study was used in this section to establish the survival of LAB. Four bacterial strains *Lactobacillus acidophilus, L. bulgaricus, L. casei* and *L. plantarum* were obtained from Anatech in Gauteng Province, South Africa and Quantum Biotechnologies in Cape Town, South Africa. API 50 CH system was obtained from BioMerieux, Johannesburg, South Africa

4.2.2 Production of BGN flour

The red BGN seeds were milled into flour using a hammer mill (Bauermeister Bauermeister Inc., Vernon Hills) with a sieve size 250 µm (No. 60), packed in clear plastic bag and stored in a refrigerator at 4-6^{o}C, till needed.

4.2.3 Production of BGN milk

The red variant BGNM was prepared as indicated in Figure 4.1 following the method reported in the previous section 3.2.3, page 51.

4.2.4 Culture validation and subculturing

Confirmation of the starter culture was done using the API 50 CH system, following the manufacturer's instructions. The pure starter cultures (0.0105 g/100 mL) were activated to make the mother cultures. Mother cultures had a bacterial load of 1.7 x 10^8 cfu mL^{-1} and 1.0 x 10^8 cfu mL^{-1} for *L. bulgaricus* and *L. plantarum* respectively, subculturing was made in BGNM (20 mLL^{-1}) and kept in a refrigerator at 4-6^oC, till needed, subculturing was done every seven days.

4.2.5 Experimental design and data analysis for best LAB strain

A three-factor factorial design (4 × 3 × 3) consisting of probiotics (*L. acidophilus, L. bulgaricus, L. casei and L. plantarum*), temperature (35, 40, 45^oC) and fermentation time (6, 15, 24 h) were used to determine the optimal time, temperature and probiotic for optimal production of BGN probiotic beverage. The independent variables and their levels are detailed in Table 4.1. The outline of experimental design for each LAB with the coded levels is given on Table 4.2. Each design point was performed in duplicate and the experiment carried out in randomised order. Dependent variables were optical density (OD), pH and titratable acidity (TTA). The experimental data was fitted to a polynomial regression model (equation 4.1) for predicting individual responses.

…… Equation 4.1

Where and are intercepts, linear, quadratic and interaction regression coefficient terms, respectively, and are independent variables time and temperature, respectively. Statistical significance of the terms in the regression equation was examined by analysis of variance (ANOVA) for each response. Lack of

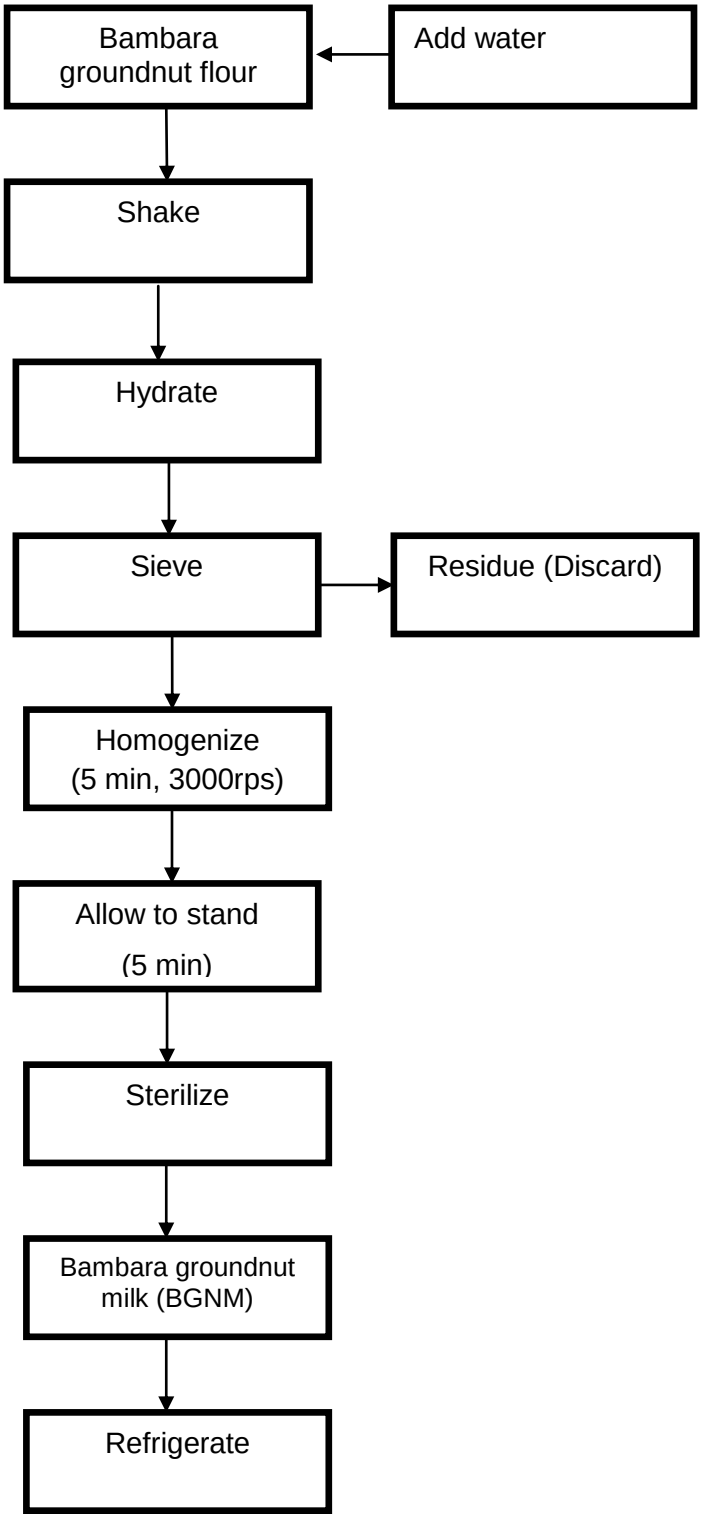

Figure 4.1 Production process for BGNM

Table 4.1 Process variables used in the three factor factorial design for optimum incubation time and temperature for the LAB strains

		Coded Variable level (x_i)		
Variable	Symbol	-1	0	+1
Temp (^oC)	X_1	35	40	45
Time (hr)	X_2	6	15	24

Transformation of coded variables (x_i) to uncoded variable (X_i) levels could be obtained from $X_1 = 5x_1 + 40$; $X_2 = 9x_2 + 15$.

Table 4.2 Independent variables and levels used for the three-factor factorial arrangement for lactic acid bacteria survival and growth[1]

Test run	X_1	X_2
1	-1	-1
2	0	-1
3	+1	-1
4	-1	0
5	0	0
6	+1	0
7	-1	+1
8	0	+1
9	+1	+1

[1]Coded levels of temperature (-1, 0, +1) correspond to 35, 40 and 45^{o}C respectively that of time (-1, 0, +1,) correspond to 6, 15 and 24 h, respectively.

fit and adequate precision was used to judge adequacy of model fit (Design-Expert 8).. The fitted model was used to navigate the design space and search for optimum incubation time, temperature and probiotic for optimum BGN probiotic beverage (BGNPB) production.

4.2.6 Fermentation of BGNM with LAB

The sterilised BGNM (60 mL) was allowed to cool to 35^{o}C, and inoculated with 2 mL of the mother culture. The sample bottles were thoroughly mixed using a Super-mixer VM-300 vortex and incubated following the time and temperature combination shown in Table 4.1. Following the same procedure each of the LAB was used. The BGNM and the probiotic mixture was incubated at 35, 40 and 45^{o}C (Memmert incubator, Lasec, South Africa) and was sampled at the designated time (Table 4.1) for viability of the strains, lactic acid production and pH.

4.2.7 Viability of strains

Viability of strains was measured as absorbance at 600 nm using a UV/VIS Spectrometer (PerkinElmer Lambda 25) according to Novak *et al.* (2009) method. BGNM without the probiotic culture was used as blank.

4.2.8 Lactic acid production

Titratable acidity was determined according to the South African legislation on dairy products (Anon, 1997). Fermented BGNM (9 ml) was pipetted into an Erlenmeyer flask. To it 0.5 mL of a 1.6% phenolphthalein indicator solution in 50% ethanol was added. The fermented BGNM was then titrated against 0.1 M NaOH solution until the first tinge of pink which persists for 30 seconds appeared. To express the titratable acidity of the yoghurt as the percentage of lactic acid, the number of millilitres of 0.1 M NaOH used in the test was divided by 10.

$$\frac{\qquad\qquad}{\qquad\qquad} \qquad \text{.....Equation 4.2}$$

4.2.9 Production of probiotic beverage

BGNM (100 mL x 2) in 250 mL schott bottles was warmed to 45^{o}C in a water bath. The BGNM was then inoculated with 3% of *L. bulgaricus* and another with *L. bulgaricus* and *L. plantarum* (1:1) and incubated in a Memmert incubator at 35^{o}C for 24 h being the optimal time estimated from section 4.2.5. The *L. bulgaricus* and *L. plantarum* were the optimum LAB as determined from section 4.2.6. The fermented BGNM was then cooled in an ice bath and stored in refrigerator at 4 ± 2^{o}C as bambara groundnut probiotic beverage (BGNPB).

For the sensory evaluation 2000 mL of the two beverages was made, divided into two parts, the first part was labelled plain, while the other was sweetened with 2.5% sucrose and flavoured with 0.01% vanilla essence. Four variants of the beverage were thus produced. The same was done for the second sample. The four samples were (1) *L. bulgaricus* plain; (2) *L. bulgaricus* and *L. plantarum* plain (3) *L. bulgaricus* sweetened and (4) *L. bulgaricus* and *L. plantarum* sweetened.

4.2.10 Quality assessment of the BGNPB

Probiotic bacterial load of the BGNPB

The probiotic bacterial load was determined according to the method described by Wang *et al.* (2007). BGNPB, L. *bulgaricus* plain and *L. bulgaricus* & *L. plantarum* plain, (1.0 mL each) were mixed with 9.0 mL of 0.1% sterile peptone solution, serial dilution up to nine were made. Dilution (3, 6 & 9) (1.0 mL) were plated out on 90 mm Petri dishes and poured MRS agar (12 ± 2 mL) (Anatech, South Africa). When the plates had set they were double layered with MRS agar (9 ± 2 mL), placed in an anaerobic jar and incubated at 37^{o}C for 24 h.

Water holding capacity

Water holding capacity (WHC) was determined according to the method of Isanga & Zhang (2009). The yoghurt was subjected to centrifugation at 2991 g for 15 minutes at a temperature of 4^{o}C using Beckman Model J2-21M Induction drive. The following formula was used to calculate WHC:

..... Equation 4.3

where W_1 = weight of yoghurt after centrifugation, W_2 = yoghurt weight
All measurements were performed in triplicates.

Susceptibility to syneresis

Susceptibility to syneresis (STS) was determined using the method of Isanga & Zhang (2009), by filtering 100 mL yoghurt through a Whatman No. 2 filter paper supported on a funnel. After 6 hours of draining, the volume of the whey collected in the beaker was measured and used as an index of syneresis. The following formula was used to calculate STS:

..... Equation 4.4

where V_1 = volume of whey collected after drainage; V_2 = volume of yoghurt sample

Apparent viscosity

The apparent viscosity and shear rate of the yoghurt was measured at 10.6^{o}C (Isanga & Zhang, 2009) using a Brookfield DV-III programmable rheometer with spindle number 61, Elscolab, Nederland. The measurements were done varying the shear rate from 1.5 to 51 s^{-1} and the corresponding viscosity was measured, in triplicates.

pH

The pH of the five BGNPB (20 mL) was measured at room temperature (21 ± 2^{o}C) using a Knick pH-Meter 766 meter, Elscolab, Nederland.

4.2.11 Sensory analysis of the BGNPB

A 40-member panel consisting of staff and students from Cape Peninsula University of Technology were served the four samples of the BGNPB. Each sample (40 mL) was identified by a three-digit random numbers and was served cold (4 - 6^{o}C) in a clear atcha tubs and a well-ventilated and naturally lighted room. Cold tap water was

provided to reset the palate. The panellists were required to evaluate each sample and rate their preference based on colour, aroma, taste, consistency and overall acceptability. The rating was on a four-point numerical scale labelled as 1- very undesirable, 2- moderately undesirable, 3- moderately desirable and 4- very desirable.

4.2.12 Data analysis

All results were reported as mean of three independent trials. Analysis of variance (ANOVA) was used to determine the differences between treatments. Duncan's multiple range tests was used to separate means where differences existed (IBM SSPS, 2010). Hierarchical cluster analysis was used to determine the number of clusters inherent in sensory data. Principal component analysis was used to extract the components that explained the variability in the data. K-mean cluster analysis was used to identify the characteristics of the clusters (IBM SPSS, 2010).

4.3 Results and discussions

4.3.1 Modelling the effect of time and temperature on BGNM

The mean responses of pH, probiotic growth and lactic acid during fermentation of BGNM are indicated in Tables 4.3a and 4.3b. The effect of temperature (X_1) and time (X_2) on pH, probiotic growth and lactic acid production for each probiotic was modelled with quadratic polynomial. The analysis of variance for the response surface quadratic model indicated that the model was significant ($F(7.57, 0.11) = 71.60$; $p = <0.0001$) in explaining the variation of probiotic, temperature and time on the pH of BGNM. The predicted R^2 of 0.81 was in reasonable agreement with the adjusted R^2 of 0.82. Furthermore, the adequate precision of 29.61 indicates an adequate signal. Hence the model with coefficient of variation (CV) of 5.70% was judged adequate to navigate the design space. The effects of probiotics ($F(12.31, 0.11) = 116.37$; $p = <0.001$) and time ($F(49.44, 0.11) = 467.40$; $p = <0.0001$) were significant on the pH of the BGNM. However, temperature had no significant effect ($F(0.054, 0.11) = 0.51$; $p = 0.4767$) on the pH of BGN.

Table 4.3a Response variables as affected by probiotics, temperature and time on BGNM during fermentation

Independents		Dependents*		
Temperature (^{o}C)	Time (h)	pH	OD	Lactic acid (%)
L. acidophilus				
35	6	6.46 ± 0.01	0.1253 ± 0.0120	0.7700 ± 0.0141
40	6	6.46 ± 0.02	0.1253 ± 0.0205	0.8200 ± 0.0141
45	6	6.47 ± 0.01	0.1054 ± 0.0006	0.9050 ± 0.0354
35	15	6.21 ± 0.014	0.0736 ± 0.0258	0.9750 ± 0.0071
40	15	6.23	0.0340 ±0.0117	0.9850 ± 0.0071
45	15	5.88 ± 0.01	0.0462 ± 0.0157	1.0950 ± 0.0071
35	24	5.58 ± 0.01	0.1726 ± 0.0090	1.1950 ± 0.0071
40	24	5.02 ± 0.08	0.1487 ± 0.0598	1.3050 ± 0.0212
45	24	4.70 ± 0.02	0.2681 ± 0.0882	1.4700 ± 0.0141
L. bulgaricus				
35	6	6.16 ± 0.01	0.1247 ± 0.0104	0.9900 ± 0.0283
40	6	5.88 ± 0.03	0.1118 ± 0.0159	1.2150 ± 0.0212
45	6	5.95 ± 0.06	0.1080 ± 0.0029	1.5000 ± 0.0141
35	15	5.32 ± 0.01	0.1718 ± 0.0180	1.1450 ± 0.0212
40	15	4.87 ± 0.01	0.1946 ± 0.0020	1.4600 ± 0.0141
45	15	4.79	0.1477 ± 0.1544	1.6350 ± 0.0212
35	24	4.84 ± 0.01	0.2078 ± 0.0144	1.3000 ± 0.0141
40	24	4.47 ± 0.01	0.2542	1.6750 ± 0.0212
45	24	4.30 ± 0.01	0.2840 ± 0.0305	1.7950 ± 0.0212

*****OD = optical density at 600 nm

Table 4.3b Response variables as affected by probiotics, temperature and time on BGNM during fermentation

Independents		Dependents*		
Temperature(^{o}C)	Time(h)	pH	OD	Lactic acid (%)
L. casei				
35	6	6.52 ± 0.02	0.1007 ± 0.0484	0.9850 ± 0.0071
40	6	6.50	0.0694 ± 0.0005	1.0000
45	6	6.50 ± 0.01	0.0944 ± 0.0163	1.0150 ± 0.0212
35	15	6.20 ± 0.3	0.1086 ± 0.0825	0.6450 ± 0.0071
40	15	6.42 ± 0.01	0.0568 ± 0.0141	0.7750 ± 0.0071
45	15	6.46 ± 0.01	0.0223 ± 0.0256	0.8150 ± 0.0071
35	24	6.02 ± 0.04	0.1003 ± 0.0153	0.9850 ± 0.0071
40	24	6.36	0.0804 ± 0.0097	0.9450 ± 0.0071
45	24	5.58 ± 1.15	0.1737 ± 0.1450	1.300 ± 0.4808
L. plantarum				
35	6	6.25 ± 0.01	0.0117 ± 0.0041	0.6500 ± 0.0707
40	6	6.24 ± 0.04	0.0384 ± 0.0016	0.3500 ± 0.0707
45	6	6.38 ± 0.01	0	0.2000
35	15	4.74 ± 0.06	0.0328 ± 0.0033	1.0000 ± 0.4243
40	15	4.70 ± 0.01	0.0458 ± 0.0016	1.7000 ± 0.1414
45	15	6.14 ± 0.25	0	0.4000 ± 0.1414
35	24	4.42 ± 0.02	0.2535 ± 0.0120	2.2500 ± 0.0707
40	24	4.38 ± 0.02	0.1866 ± 0.0605	1.7500 ± 0.0707
45	24	6.08 ± 0.25	0.0492 ± 0.0423	1.5500 ± 0.0707

*****OD = optical density at 600 nm

The two way interaction of probiotics and temperature (F (4.31, 0.11) = 40.78; p = <0.0001); probiotics and time (F (1.78, 0.11) = 16.81; p = <0.0001) significantly affected the pH of BGNM. Interaction means that the effect produced on pH of BGNM by a change in probiotics depends on the level of fermentation time and temperature. The quadratic effect of temperature was significant (F (0.55, 0.11) = 5.16; p = 0.0242) on the pH of BGNM. However, the quadratic effect of time was not significant (F (1.89, 0.11) = 1.89; p = 0.1705).

The effect of temperature (X_1) and time (X_2) for each probiotic on viability as OD was modelled with quadratic polynomial. The analysis of variance for the response surface quadratic model indicated that the model was significant (F (0.048, 0.0030) = 15.98; p = <0.0001) with significant lack of fit. However, the predicted R^2 of 0.54 was in reasonable agreement with the adjusted R^2 of 0.59. Furthermore, the adequate precision of 18.56 indicates an adequate signal. Hence the model was judged adequate to navigate the design space. The effect of probiotics (F (0.083, 0.0030) = 27.73; p = <0.0001) and time (F (0.23, 0.0030) = 78.33; p = <0.0001) were significant on the OD of the BGNM. However, temperature had no significant effect (F (0.0010, 0.0030) = 0.33; p = 0.5687) on the OD of the BGNM. The two way interaction of probiotics and temperature (F (0.019, 0.003) = 6.44; p = <0.0004); probiotics and time (F (0.018, 0.003) = 6.18; p = <0.0006) significantly affected the OD of BGNM, while that of temperature and time had no significant effect (F (0.0057, 0.003) = 1.92; p = 0.1683) on the OD of the BGNM. Interaction means that the effect produced by a change in fermentation temperature on probiotic growth in BGNM depends on the level of fermentation time. The quadratic effect of time was significant (F (0.066, 0.003) = 22.07; p = 0.0001) on the OD of BGNM. However, the quadratic effect of temperature was not significant (F (0.0005, 0.003) = 0.16; p = 0.6890).

The effect of temperature (X_1) and time (X_2) for each probiotic for lactic acid content was modelled with quadratic polynomial. The analysis of variance for the response surface quadratic model indicates that the model was significant (F (0.85, 0.038) = 22.21; p = <0.0001) with significant lack of fit. However, the predicted R^2 of 0.77 was in reasonable agreement with the adjusted R^2 of 0.81. Furthermore, the

adequate precision of 22.84 indicates an adequate signal. Hence the model with coefficient of variation (CV =17.32%) was judged adequate to navigate the design space. The effects of probiotics (F (0.73, 0.038) = 19.29; p = <0.0001) and time (F (4.22, 0.038) = 111.05; p = <0.0001) were significant on the lactic acid content of the BGNM. However, temperature had no significant effect (F (0.052, 0.038) = 1.37; p = 0.2472) on the lactic acid content of the BGNM. The two way interaction of probiotics and temperature (F (0.63, 0.038) = 16.61; p = <0.0001); probiotics and time (F (1.07, 0.038) = 28.07; p = <0.0001) significantly affected the lactic acid content of BGNM, while that of temperature and time had no significant effect (F (0.0032, 0.038) = 0.084; p = 0.7728) on the lactic acid concentration of the BGNM. Interaction means that the effect produced by a change in fermentation temperature on lactic acid content of BGNM depends on the level of fermentation time. The quadratic effect of time was significant (F (0.20, 0.038) = 5.17; p = 0.0268) on the lactic acid content of BGNM. However, the quadratic effect of temperature was not significant (F (0.054, 0.038) = 1.41; p = 0.2398).

4.3.2 Effect of incubation temperature and time on pH of BGNM with respect to different probiotics

The main effect of probiotics, temperature and time on the pH of BGNM during fermentation is detailed in Table 4.4. *L bulgaricus* significantly reduced the pH of BGNM to 5.18. There was no significant difference in temperature on pH. Fermentation for 24 h significantly reduced the pH to 5.14. The model parameters for the effect of incubation temperature and time for each strain as estimated by the quadratic polynomial model are indicated in Table 4.5. The pH response surface for *L. acidophilus, L. bulgaricus, L. casei and L. plantarum* as affected by incubation time and temperature are illustrated in Figures 4.2; 4.3; 4.4 and 4.5 respectively. For all the probiotics, temperature, time and their two way interaction (X_1X_2) had a negative effect on the pH of the BGNM (Table 4.5). Hence, as the temperature and time increased a drop in pH was observed (Figures. 4.2; 4.3; 4.4 and 4.5). The negative main

Table 4.4 Effect of probiotics, temperature and time on BGNM[1,2]

	pH	OD	Lactic acid (%)
Probiotics			
L. acidophilus	5.89 ± 0.64[a]	0.1221 ± 0.0746[a]	1.0578 ± 0.2242[a]
L. bulgaricus	5.18 ± 0.66[b]	0.1783 ± 0.0720[b]	1.4128 ± 0.2608[b]
L. casei	6.28 ± 0.41[c]	0.0896 ± 0.0590[a]	0.9406 ± 0.2139[c]
L. plantarum	5.48 ± 0.86[d]	0.0625 0.0949[a]	1.0944 ± 0.7280[a]
Temperature (^{o}C)			
35	5.73 ± 0.71[a]	0.1236 ± 0.0715[a]	1.0742 ± 0.4216[a]
40	5.63 ± 0.84[a]	0.1121 ± 0.0723[a]	1.1650 ± 0.4229[a]
45	5.77 ± 0.79[a]	0.1036 ± 0.1116[a]	1.1400 ± 0.4974[a]
Time (h)			
6	6.32 ± 0.22[a]	0.0810 ± 0.0534[a]	0.8667 ± 0.3451[a]
15	5.66 ± 0.70[b]	0.0768 ± 0.0737[a]	1.0525 ± 0.3939[b]
24	5.14 ± 0.77[c]	0.1816 ± 0.0857[b]	1.4600 ± 0.3771[c]

[1]OD = optical density at 600 nm

[2]BGNM = Bambara groundnut milk

Table 4.5 Regression coefficients of predicted quadratic polynomial model of pH for each probiotic[a,b]

Coefficients	Probiotic			
	L. acidophilus	*L. bulgaricus*	*L. casei*	*L. plantarum*
Linear				
b_0	15.4510*	14.9037*	13.9097*	9.2025*
b_1	-0.3783	-0.3807	-0.3480	-0.2334
b_2	-0.0998*	-0.1038*	-0.0513*	-0.0959*
Interaction				
b_{12}	-3.7248×10^{-5}	-3.7244×10^{-5}	-3.7247×10^{-5}	-3.7247×10^{-5}
Quadratic				
b_{11}	4.2406×10^{-3}*	4.2406×10^{-3}*	4.2406×10^{-3}*	4.2406×10^{-3}*
b_{22}	8.0142×10^{-4}	8.0142×10^{-4}	8.0142×10^{-4}	8.0142×10^{-4}

[a] where X_1 = Temperature, X_2 = Time.

[b] * = Significant at p = 0.05

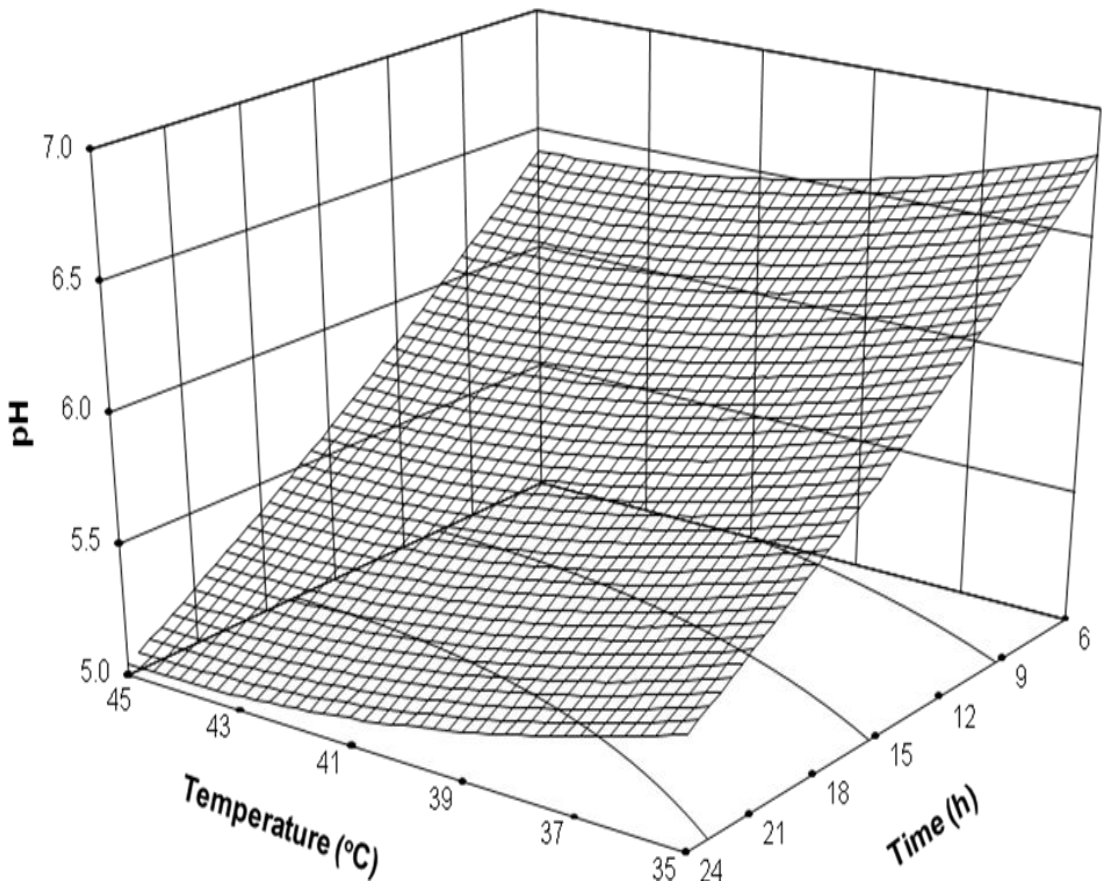

Figure 4.2 Effect of incubation time and temperature on pH of BGNM for *L. acidophilus*

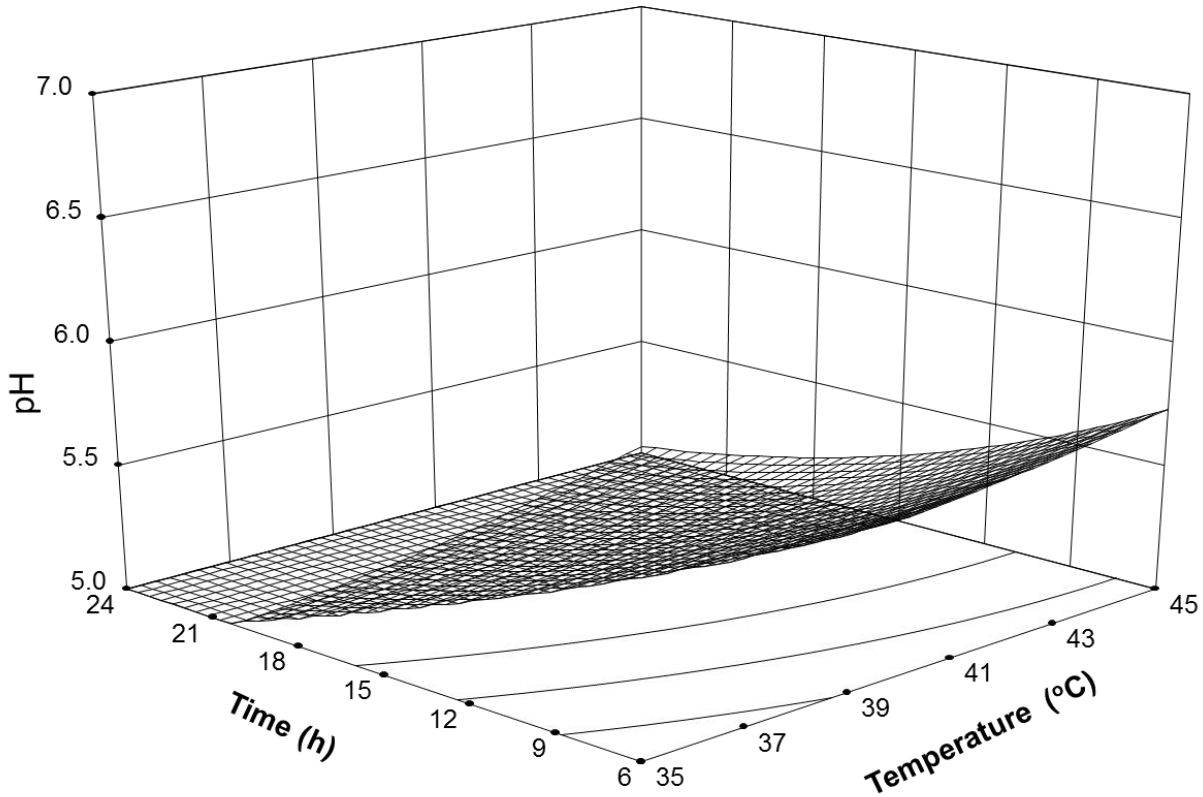

Figure 4.3 Effects of time and temperature on the pH of BGNM for *L. bulgaricus*

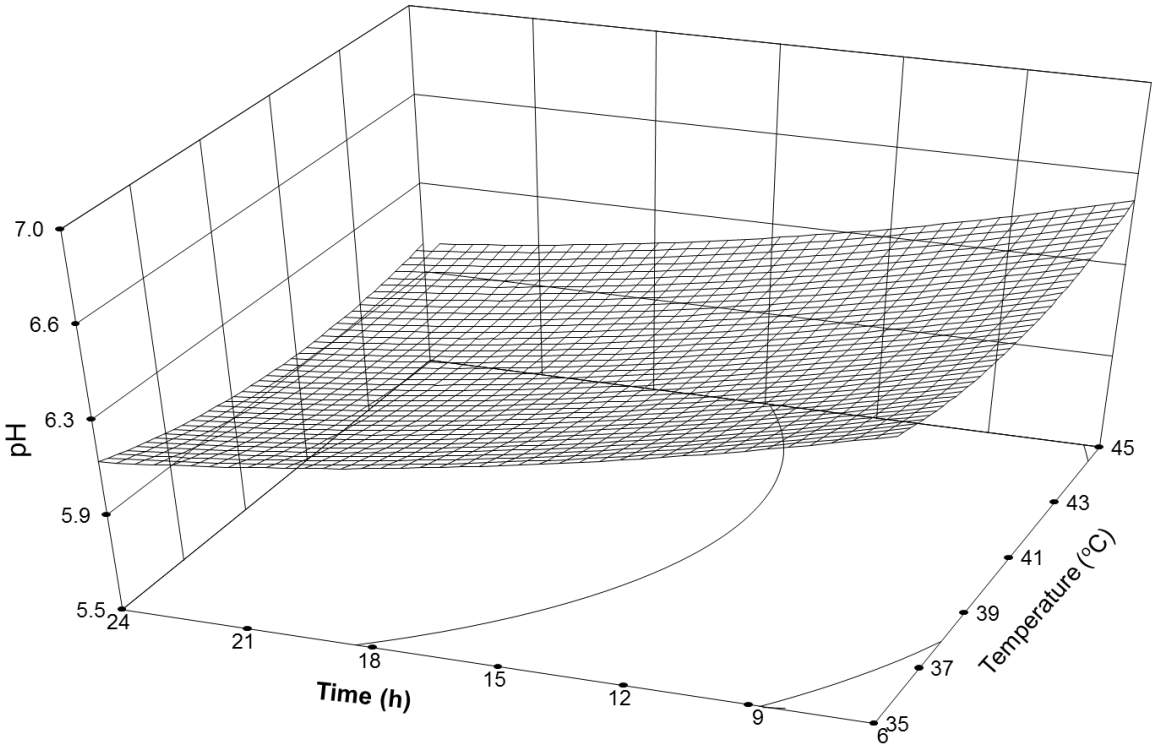

Figure 4.4 Effect of time and temperature on the pH of BGNM for *L. casei*

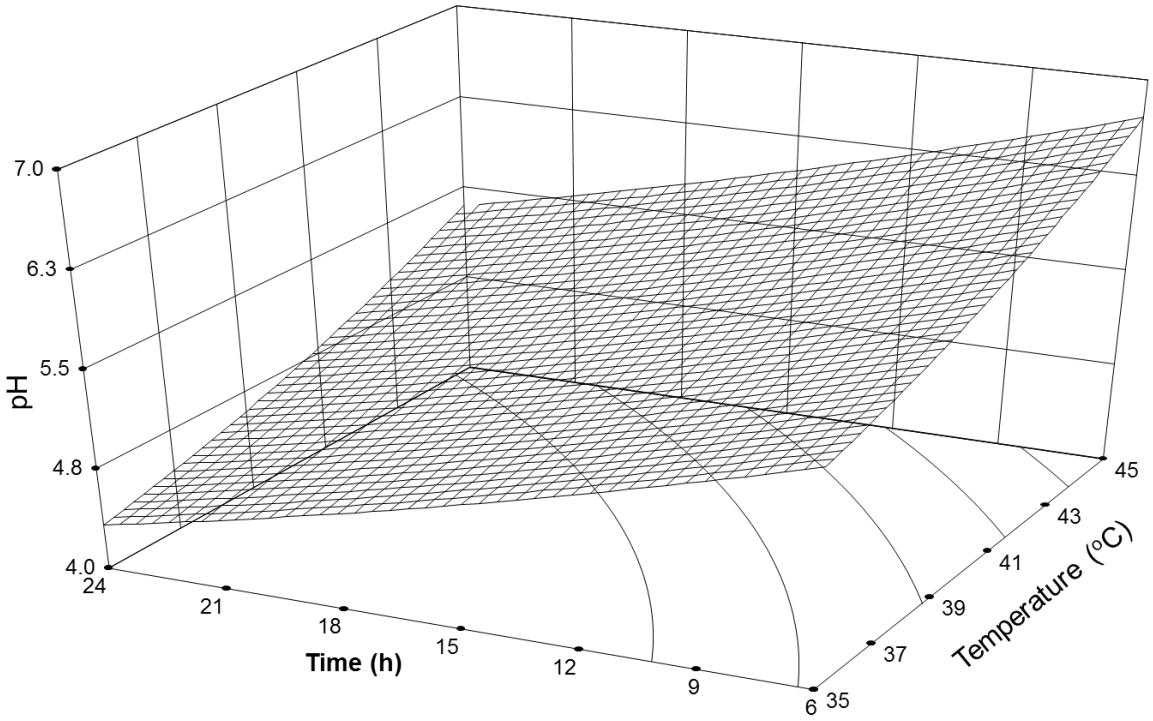

Figure 4.5 Effect of time and temperature on the pH of BGNM for *L. plantarum*

effects for time and temperature and the positive quadratic effect in each case resulted in a convex curve. This implied that there was a minimum turning point beyond which the effect of time and temperature pH.

Figure 4.2 shows the response surface for pH of BGNM based on the incubating temperature and time for the growth of *L acidophilus*. There was a significant ($p < 0.05$) drop in pH, as the incubation time increased from time 6 h to 24 h (Table 4.5 Figure 4.2). However, there was no significant drop in pH as the temperature was increasing from 35^{o}C to 45^{o}C. The contour line hinged on the time axis further confirmed that time had the most significant effect on the pH of BGNM. The response surface was saddle shaped, where a minimum or maximum of the response variable could be found at various combinations (Jideani & Onwubali, 2009). Similar trend was observed for *L. bulgaricus, L. casei and L. plantarum* (See Figures 4.3, 4.4 and 4.5).

The pH of yoghurt type product is 4.2-4.6, acidification patterns depend on the sugar assimilation properties of the probiotics. Probiotics affect the pH of their medium through their growth and metabolism. However their growth is strain specific, different strains have an effect on the medium in different ways due to their different feeding patterns as well as growth requirement. In the present study the four LAB had an effect on pH but at different levels. This observation was in agreement with that of Oliveria *et al*. (2001) who stated that strain linked variability of acidification rate revealed sharp differences between strains in the rate of acid production on carbohydrates found in soy. Therefore, for pure cultures strain selection is essential to obtain adequate acidification rates.

Temperature affects the growth and metabolism rates of probiotics. Optimum temperature of probiotics is also strain specific (Oliveria *et al*, 2001). However, in this study no significant effect of temperature was observed on the pH. The implication was that any of the temperatures in the range of the study (25-45^{o}C) can effectively be used to incubate the probiotics in BGNM.

Fermentation time required to attain a pH below 4.5 is typically 10 hours or more at 37^{o}C (Oliveria *et al*., 2001). However, in this study 24 hours was required to achieve that pH. Perhaps the probiotics needed more time to assimilate the sugars

present in the BGNM, or the inoculation load was very low. Increasing fermentation time increases the microbial load. Bacterial growth results in increase in organic acids especially lactic acid, which is by product of bacterial metabolism. Conversely, a drop in pH is an indication of growth. In this study significant steady drop in pH was observed, indicating an increase in the microbial load. These results are in agreement with the observation of Wang *et al*. (2007), where the pH of *L. plantarum* inoculated peanut flour declined from 6.59 to 3.98 over a period of 72 hrs. In the study by Angelov *et al*. (2005) a pH of 4.52 was attained within 8 hour where oatmeal was fermented using *L. plantarum* B28. In the study by Oliveria *et al.* (2001) time to achieve a pH of 4.5 varied from 2.1 to 14.3 hours for the four probiotics analysed. Industrial short fermentation times are preferable in order to increase plant output as well as to reduce unwanted contaminating microorganisms. The pH of between 4.0 and 4.5 attained in this study was adequate, according to Yoon *et al*. (2004). The pH in this range suppresses the development of contaminating microorganisms flora and on the other hand, organoleptically appropriate (McMaster *et al.*, 2005).

4.3.3 Effect of incubation temperature and time on the growth of probiotics in BGNM

The main effect of probiotic, temperature and time on the growth of lactic acid bacteria in BGNM is indicated in Table 4.6. Incubation for 24 h significantly increased the viability of the bacteria with high absorbance of 0.1816 ($\approx 10^7$ cfu/mL). However, there was no significant effect of temperature on the survival of the lactic acid bacteria. The growth rate of *L. bulgaricus was* significantly higher (0.1783) compared to the others. The model parameters for the effect of incubation temperature and time for each strain are indicated in Table 4.6. The OD response surface for *Lactobacillus acidophilus, L. bulgaricus, L. casei and L. plantarum* as affected by incubation time and temperature are illustrated in Figures 4.6, 4.7, 4.8 and 4.9, respectively.

Temperature and time had a negative effect on the growth of each of the probiotics. Hence, as the temperature and time were increasing a drop in OD was observed (Figures 4.6, 4.7, 4.8, 4.9 and Table 4.6).

Table 4.6 Regression coefficients of predicted polynomial model of OD for *L. acidophilus, L. bulgaricus, L. casei* and *L. plantarum*[a,b]

	Dependent variables			
Coefficients	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
Linear				
b_0	0.3618*	0.4596*	0.5284*	0.7639*
b_1	-0.0112	-0.0136	-0.0153	-0.0241
b_2	-0.0210*	-0.0174*	-0.0233*	-0.0168*
Interaction				
b_{12}	2.0732×10^{-4}	2.0732×10^{-4}	2.0732×10^{-4}	2.0732×10^{-4}
Quadratic				
b_{11}	1.5305×10^{-4}	1.5305×10^{-4}	1.5305×10^{-4}	1.5305×10^{-4}
b_{22}	5.5853×10^{-4}*	5.5853×10^{-4}*	5.5853×10^{-4}*	5.5853×10^{-4}*

[a] where X_1 = temperature, X_2 = time.

[b] * = Significant at p = 0.05

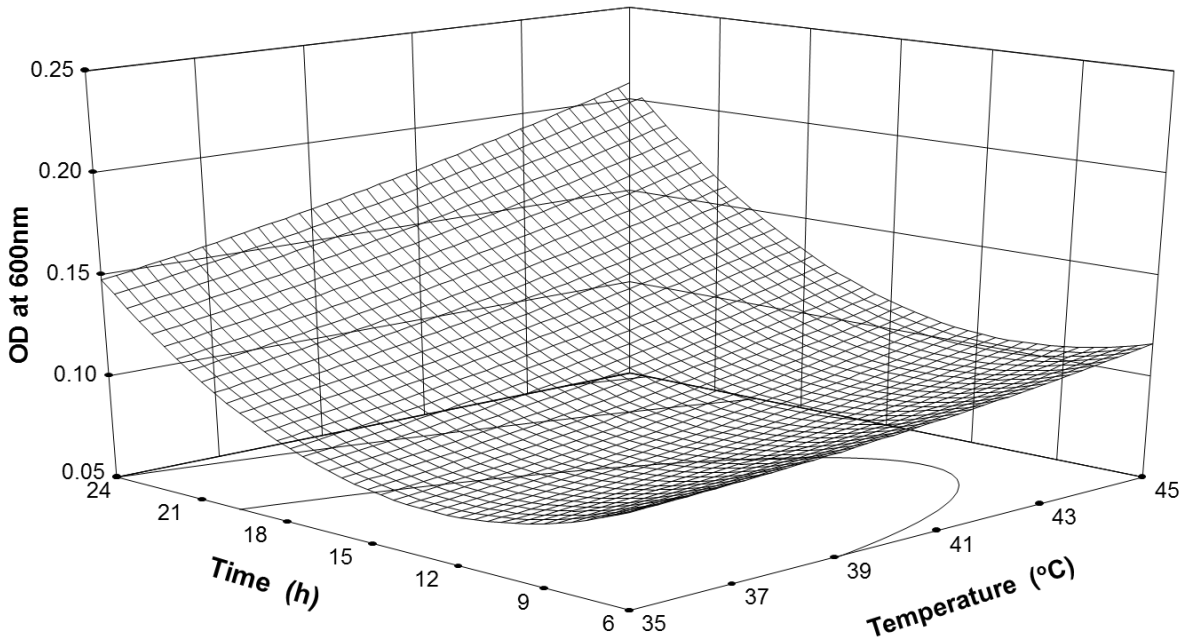

Figure 4.6 Effects of time and temperature on the OD of *L. acidophilus*

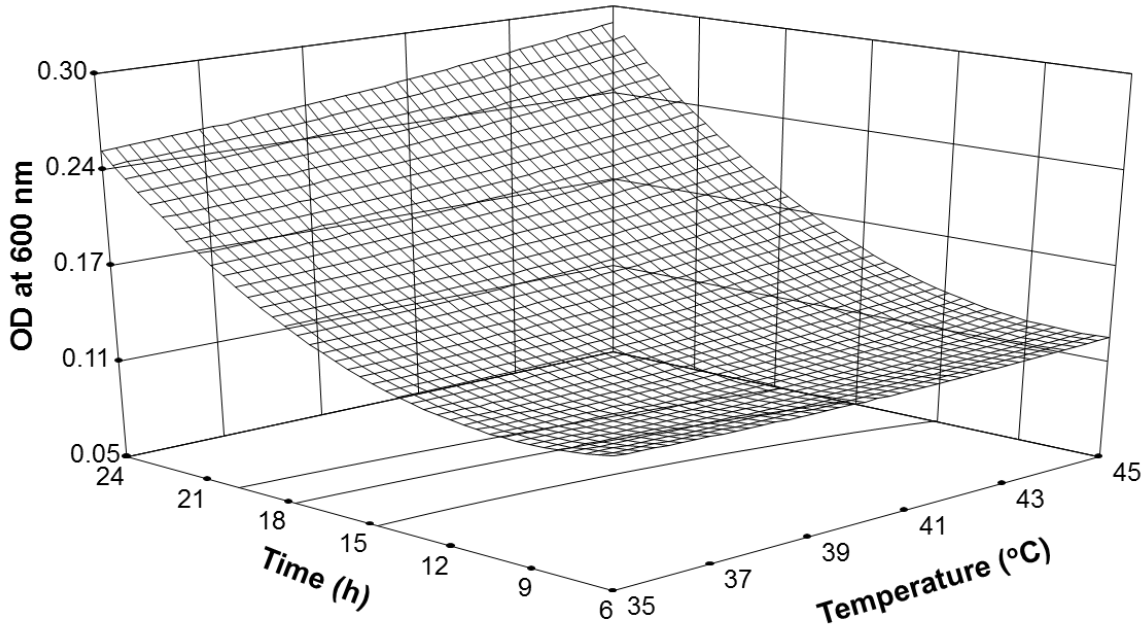

Figure 4.7 Effects of time and temperature on the OD of *L. bulgaricus*

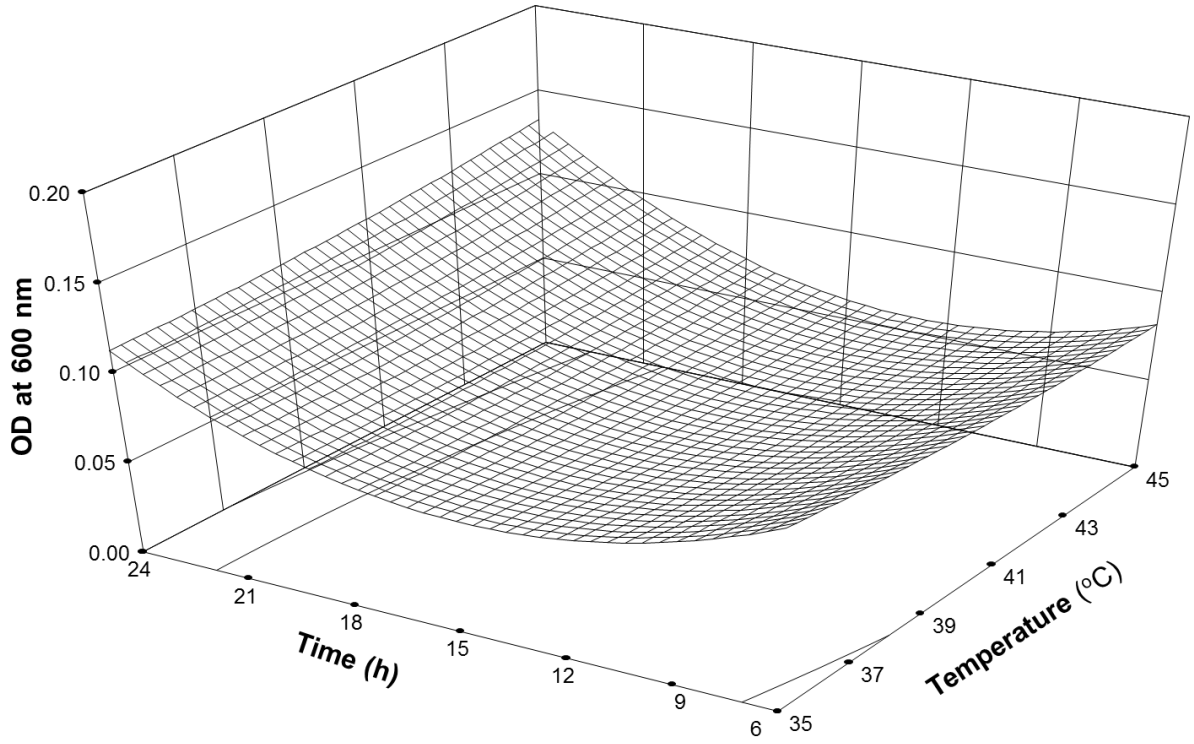

Figure 4.8 Effects of time and temperature on the OD of *L. casei*

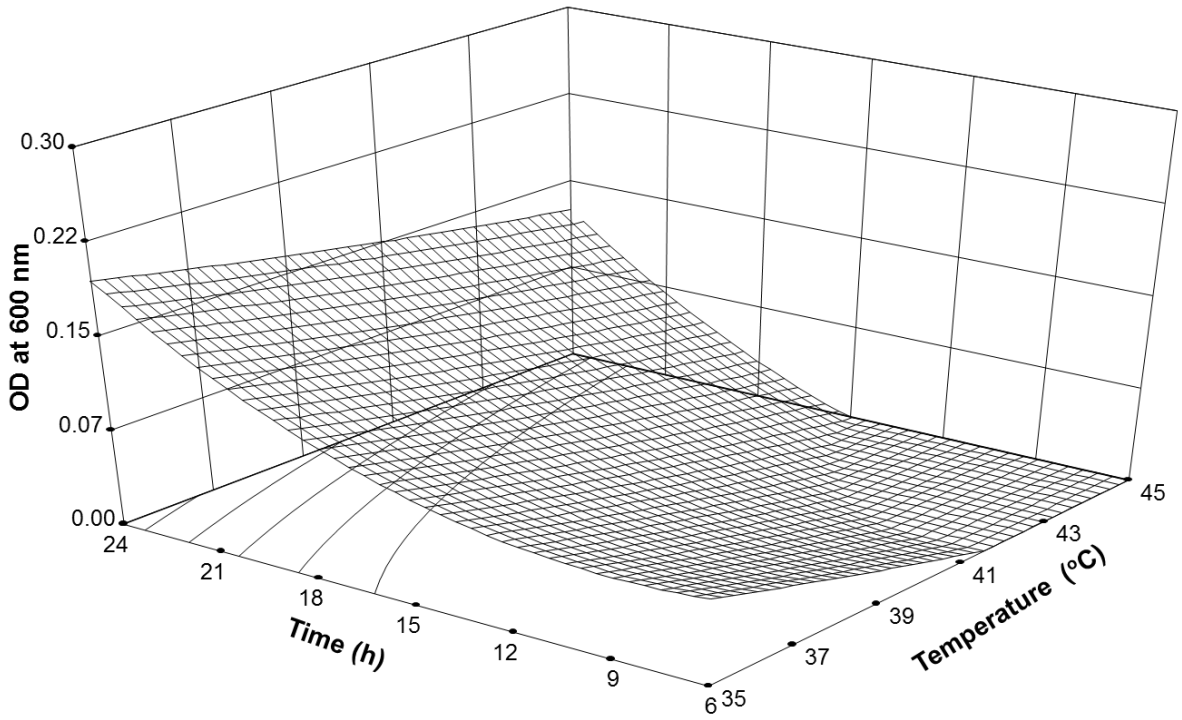

Figure 4.9 Effects of time and temperature on the OD of *L. plantarum*

The two way interaction of temperature and time as well as the quadratic effect of temperature and time both had a positive effect on the growth and survival of the probiotics in BGNM (Table 4.6). The negative linear terms and the positive quadratic term for time and temperature in each case indicated that the decrease in growth was more than linear because the quadratic term was exerting a positive influence on the curve resulting in a convex curve. This implies that there was a minimum turning point beyond which the effect of time and temperature increases the viability of the organisms. The growth and survival of the probiotics differed in the BGNM due to their physiological differences. The more the probiotic survived and grew in the BGNM the higher the OD. Novak *et al.* (2009) concluded that OD was a linear function of population density.

4.3.4 Effect of incubation temperature and time on the lactic acid production by probiotics in BGNM

The main effect of probiotics, fermentation temperature and time on lactic acid production in BGNM is detailed in Table 4.7. Significant ($p < 0.05$) differences exist between the probiotics in lactic acid production. *L. bulgaricus* significantly produced more lactic acid compared to others (Table 4.3, pages 115 & 116). There was no difference in lactic acid production between *L. acidophilus* and *L. plantarum*. Fermentation for 24 h significantly increased the lactic acid (1.46%) production. The model parameters for the effect of incubation temperature and time for each strain are indicated in Table 4.7. The lactic acid response surface for *Lactobacillus acidophilus, L. bulgaricus, L. casei and L. plantarum* as affected by incubation time and temperature are illustrated in Figures 4.10, 4.11, 4.12 and 4.13, respectively. In each case the main effect of temperature was positive, as temperature increases there was a corresponding increase in lactic acid production.

The quadratic effect of temperature in each case was negative. The positive effect of temperature and the negative effect of the quadratic effect of temperature resulted in a concave curve. The implication was that the increase in lactic acid due to positive influence of temperature was less than a linear increase since the quadratic effect was pulling the curve downward. For *L. acidophilus*, *L. bulgaricus*

Table 4.7 Regression coefficients of predicted polynomial model of lactic acid content for *L. acidophilus, L. bulgaricus, L. casei* and *L. plantarum*[a,b]

	Dependent variables			
Coefficients	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
Linear				
b_0	-3.3594*	-4.1771*	-3.1107*	-1.0813*
b_1	0.1997	0.2318	0.1991	0.1237
b_2	-0.0226*	-0.0302*	-0.0457*	0.0306*
Interaction				
b_{12}	2.2222×10^{-4}	2.2222×10^{-4}	2.2222×10^{-4}	2.2222×10^{-4}
Quadratic				
b_{11}	-2.3167×10^{-3}	-2.3167×10^{-3}	-2.3167×10^{-3}	-2.3167×10^{-3}
b_{22}	1.3683×10^{-3}*	1.3683×10^{-3}*	1.3683×10^{-3}*	1.3683×10^{-3}*

[a] where X_1 = temperature, X_2 = time.

[b] * = Significant at p = 0.05.

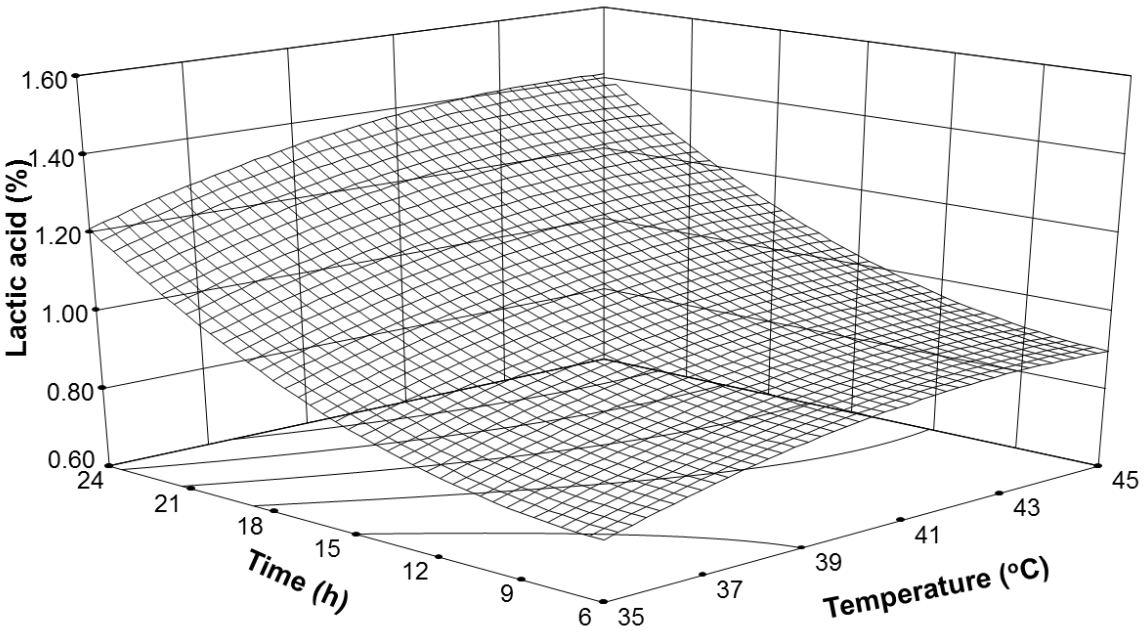

Figure 4.10 Effects of time and temperature on the lactic acid of *L. acidophilus*

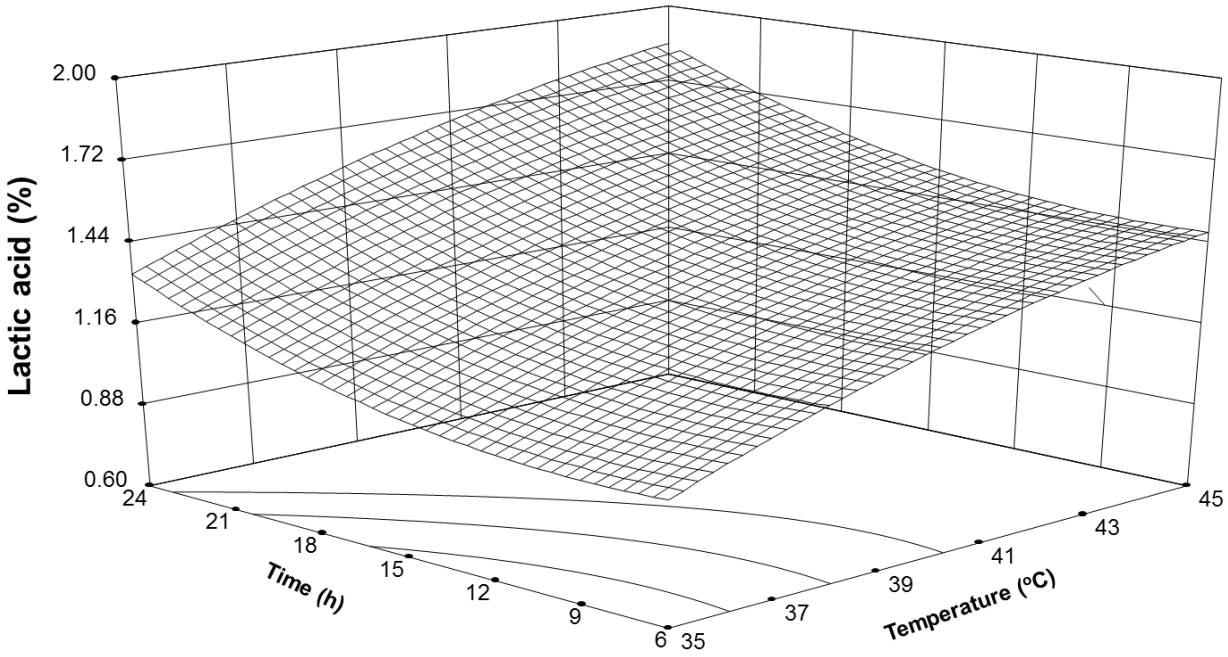

Figure 4.11 Effects of time and temperature on lactic acid of *L. bulgaricus*

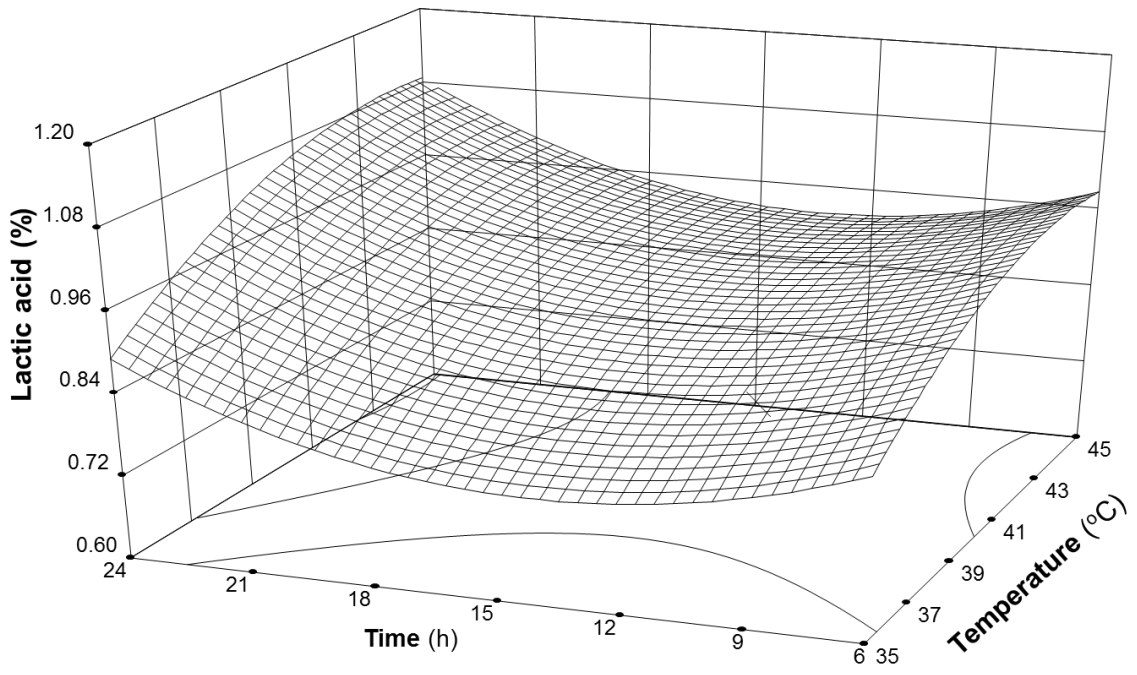

Figure 4.12 Effects of time and temperature on the lactic acid of *L. casei*

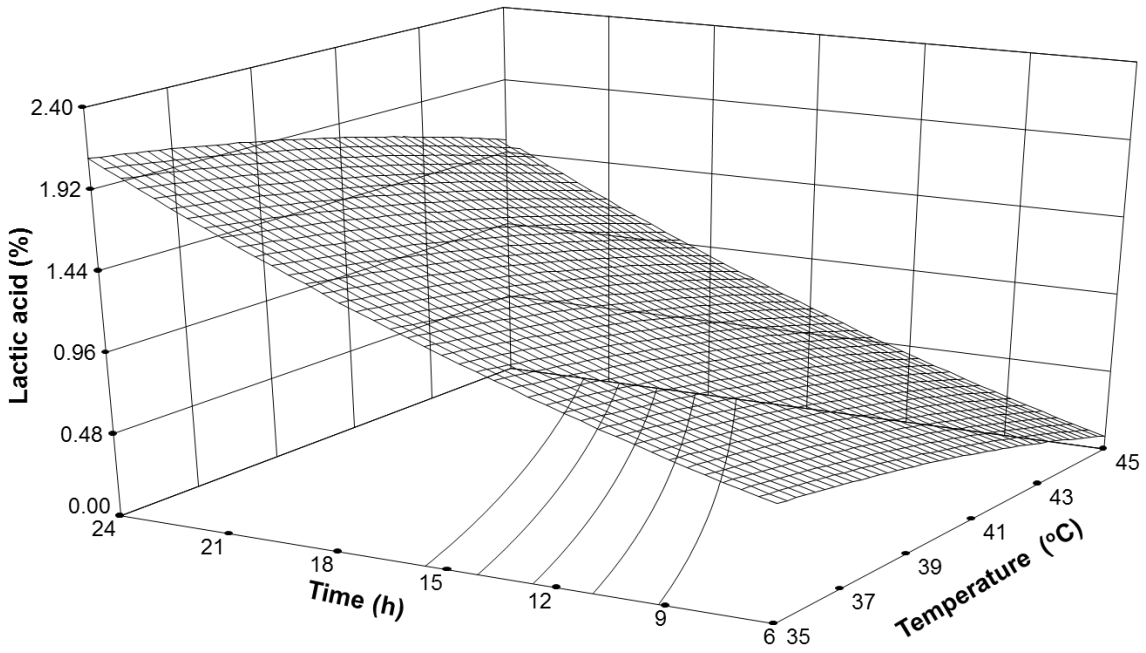

Figure 4.13 Effects of time and temperature on lactic acid of *L. plantarum*

and *L. casei* the main effect of incubation time was negative, increase in time resulted in decrease in lactic acid produced. For these organisms the negative main effect of time and the positive effect of quadratic time resulted in a convex curve. This meant that the decrease in lactic acid produced due to the negative main effect of time was more than the linear as the quadratic effect exerted an upward effect on the curve. For *L. plantarum* both the main effect of time and its quadratic effect were positive. This implied that there was a greater quadratic effect than a linear increase in the rate at which *L. plantarum* produced lactic acid in BGNM. The two way interaction of temperature and time had a positive effect on the lactic acid production in BGNM.

The four probiotics used in this study produced lactic acid at different rates, resulting in different lactic acid content. The rate of lactic acid production was a better indicator of growth, than pH, since the BGNM could have had a buffering effect (Saarela *et al*., 2006). The difference in lactic acid content amongst the four strains could be attributed to the different physiological characteristics of the probiotics in agreement with the study of Oliveria *et al*. (2001). The same range of lactic acid was reported by Angelov *et al*. (2005) & Wang *et al.* (2007). However, lower lactic acid content at pH 4.0 of LAB fermented cowpea milk was reported by Sanni *et al.* (1999). This difference may be attributed to the different substrates as well as the specific fermentation properties of the different strains. Fermentation time depends on the probiotic employed. Increasing the fermentation time increases the sugar assimilation by the probiotics, however increasing time above a certain point might decrease the viable cell count, which could be due to the high acidity in the BGNM (Yoon *et al*., 2004).

4.3.5 Optimal probiotic, fermentation temperature and time for the production of BGNPB

The goal of the optimisation was to minimize fermentation temperature and pH while maximising fermentation time and lactic acid bacteria growth (OD) within the range of the probiotics used in the study. The optimal condition for the production of BGN probiotic beverage was estimated to be *L. bulgaricus* fermented at 35^{o}C for 24 h with a desirability of 0.854. This condition was estimated to yield a pH of 4.7 with optical

density of 0.25 (8 log cfu) at 600 nm. The next promising probiotic was *L. plantarum* that could be fermented at 35^{o}C for 24 h with estimated pH of 4.4 and optical density of 0.20 (7.7 log cfu) at 600 nm with 0.843 desirability. The objective of optimising the fermentation temperature and time while identifying the best probiotic strain was achieved. The hypothesis stating that different LAB strains will show different growth and survival patterns in BGNM was accepted

4.3.6 Quality assessment of BGNPB

The tests of between subjects effects indicated that pH ($F(0.960, 3.33E\text{-}5) = 450$, $p < 0.05$) and viscosity ($F(1944, 1) = 1944$, $p < 0.05$) of the two BGNM probiotic beverage were significantly different from each other while their syneresis ($F(0.960, 4.853) = 0.198$, $p = 0.68$) and water holding capacity ($F(0.260, 1.198) = 0.217$, $p = 0.665$) did not differ significantly. Table 4.8 details the descriptive statistics of the BGNM probiotic beverage quality.

The pH of the BGNM probiotic was 4.39 and 4.29, respectively for the *L. bulgaricus* and *L. bulgaricus & L. plantarum* fermented beverage (Table 4.8). According to Yoon *et al*. (2004), *L. plantarum* had the most ability to reduce sugars thus giving a slightly lower pH than that of *L. bulgaricus* in the tomato juice fermentations, this fermentation pattern was expected for the BGNM. Strain linked variability of acidification rate (Sanni *et al*., 1999; Serra *et al*., 2009) revealed sharp differences between strains in the rate of acid production on carbohydrates found in soybean. Therefore for pure cultures, strain selection is essential to obtain adequate acidification rates.

Viscosity (Table 4.8) of the two beverages were 62% and 98%, *L.bulgaricus* and *L. bulgaricus & L. plantarum*, respectively. The significant difference could be attributed to the ability of the culture to assimulate sugars and the starch content which is relatively abandant in legumes (Goli *et al*., 1997). Assimulation of sugars resulted in an incesease in organic acids, resulting in the pH drop. Low pH favours protein denaturation, which may had lead to floculation of global proteins resulting in gel formation thus increasing the viscosity of the BGNM. BGNPB from *L. bulgaricus & L. plantarum*, was anticipated to be more viscous since *L. plantarum* was

Table 4.8 Physical characteristics of two BGN probiotic beverages[1,2]

	Physical property			
BGNPB	pH	Viscosity (%).	Syneresis (%)	WHC (%)
L. bulgaricus	4.39 ± 0.01[a]	62.0 ± 1.0[a]	39.3 ± 3.1[a]	62.8 ± 1.5[a]
L. bulgaricus & *L. plantarum*	4.29 ± 0.01[b]	98.0 ± 1.0[b]	38.5 ± 0.6[a]	62.3 ± 0.3[a]

[1]BGNPB = Bambara groundnut probiotic beverage; WHC = Water holding capacity

[2]Value are means ± standard deviation. Values with different superscripts in the same column are significantly different ($p < 0.05$).

said to have the most ability to assimulate sugars compared to *L. bulgaricus* (Yoon *et al*., 2004). Starch in the BGNM, might had gelatinised during the heat treatment, leading to the formation of a gel, which affected the consistency of the BGNM. Lee & Lucey (2010) reported that stirred yoghurts that had been incubated at low temperature (< 40^{o}C) had higher viscosity compared to yoghurts incubated at high temperatures (> 40^{o}C). Fermentation of BGNM at 35^{o}C affected the physical and microstructure of the fermented BGNM. Syneresis was 39.33% and 38.55 for *L. bulgaricus* and *L bulgaricus* & *L. plantarum* (Table 4.8) beverage, respectively, similar syneresis results were reported by Aidoo *et al.* (2010). Amatayakul *et al* (2006) reported that there was no significant difference in syneresis between three yoghurt samples with the non-exopolysaccharide (EPS), capsular-EPS and ropy-EPS added. Low total solids in the BGNM could had been responsible for the observed extent of syneresis (Amatayakul *et al.,* 2006).

The water holding capacities (Table 4.8) of the two BGNPB were 62.8% and 62.3%; respectively for *L. bulgaricus* and *L. bulgaricus* and *L. plantarum.* These results were in agreement with those of Aidoo *et al*. (2010). Water holding capacity was also depended on the protein and starch available in the BGNM to form hydrogen bonds with the water (Lee & Lucey, 2010). The water holding capacity cloud also be attributed to the acidification process because it results in the formation of a three dimensional network, which locks in the water (Lee & Lucey, 2010). Gel formation is one of the main properties in yoghurt manufacturing. Rheological properties of the gel are affected by milk composition, time and temperature, type and quantity of culture, fermentation temperature, gel breaking or not, and storage conditions until the end of the product's shelf life (Amatayakul *et al.,* 2006).

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4.3.7 Consumer acceptability of the BGNPB

The demography of the panellists is indicated in Table 4.9. There were 40 panellists 62.5% of whom were males, 55% were staff members and 5.0% were less than 20 years old, 63% within 20-30 years of age and 32.5% within 31-40 years of age.

Multivariate analysis of variance indicated that the panellists differed significantly ($p < 0.05$) in their rating for appearance, colour, aroma, taste, mouth feel

Table 4.9 Demography of the panellists*

Item	Frequency (Percentage)
Gender	
Male	25 (62.5)
Female	15 (37.5)
Status	
Staff	18 (45)
Student	22 (55)
Age category	
<20	2 (5)
20-30	25 (62.5)
31-40	10 (25)
>40	3(7.5)

*Numbers are frequency and percentage in bracket.

and overall acceptability (Table 4.10). The difference in rating amongst the panellist was expected, since people from different age groups (>20-40 yrs), different occupation (students, lecturers and non-academic staff) were recruited. The four BGNPB samples were significantly different in terms of aroma ($F(5.625, 0.621) = 9.062$, $p < 0.05$), taste ($F(20.517, 0.551) = 37.245$, $p < 0.05$), mouthfeel ($F(9.990, 0.579) = 17.243$, $p < 0.05$) and overall acceptability ($F(8.056, 0.539) = 14.942$, $p < 0.05$), while the appearance ($F(0.375, 0.0.285) = 1.315$, $p = 0.273$) and colour ($F(0.800, 0.385) = 2.0750$, $p = 0.107$) were judged to be the same. The differences in aroma, taste, mouthfeel and overall acceptability may be attributed to the sucrose which was added for tastes as well as the vanilla flavour. Adding the sweetener altered the taste and viscosity which was perceived as the mouthfeel hence the overall acceptability. The fermentation by products, being different, could have contributed to the perceived difference in taste mouthfeel and overall acceptability.

The panellists mean rating for appearance ranged from 2.6 for *L. bulgaricus* plain to 2.9 for *L. bulgaricus & L. plantarum* sweetened (Table 4.10), in specific terms, moderately desirable. BGNPB *L. bulgaricus & L. plantarum* sweetened was rated higher in appearance compared to others. Appearance which is the opacity of milk is due to its content of suspended particles of protein and certain minerals. According to Nti (2009) BGN varieties differ in their carbohydrates content. Starch gelatinisation during the sterilisation step and the denaturation of protein during fermentation resulted in the rich appearance of the milk. The difference in chemical composition as a result of the different end product of fermentation, sucrose and vanilla essence also impacted the panellist rating for appearance. Figure 4.14 shows the typical BGNPB.

In terms of colour the panellists mean rating (Table 4.10) ranged from 2.5 (moderately desirable) for *L. bulgaricus & L. plantarum* plain to 2.8 (moderately desirable) for *L. bulgaricus & L. plantarum* sweetened. The BGNPB *L. bulgaricus & L. plantarum* sweetened was rated significantly higher in colour, followed by *L bulgaricus* sweetened , with significantly low rating for *L. bulgaricus* plain and *L. bulgaricus & L. plantarum* plain. The difference between the highly rated sweetened

Table 4.10 Sensory parameters of BGN probiotic beverage[1,2]

Sensory attributes	*L. bulgaricus* (plain)	*L. bulgaricus* & *L. plantarum* (plain)	*L. bulgaricus* (sweetened)	*L. bulgaricus* & *L. plantarum* (sweetened)
Milk appearance	2.6 ± 1.0[a]	2.7 ± 1.0[a]	2.8 ± 1.0[a]	2.9 ± 0.9[a]
Milk colour	2.7 ± 0.9[a]	2.5 ± 1.0[a]	2.8 ± 1.0[a]	2.8 ± 1.0[a]
Milk aroma	2.2 ± 1.0[a]	1.8 ± 0.8[b]	2.7 ± 1.0[c]	2.4 ± 1.1[c]
Milk taste	1.5 ± 0.8[a]	1.6 ± 0.7[a]	2.9 ± 1.0[b]	2.6 ± 0.9[b]
Milk mouthfeel	1.9 ± 1.0[a]	1.8 ± 0.9[a]	2.7 ± 1.0[b]	2.7 ± 1.1[b]
Milk overall acceptability	2.0 ± 1.0[a]	1.9 ± 0.9[a]	2.8 ± 1.0[b]	2.5 ± 0.9[b]

[1]BGN – bambara groundnut

[2]Values are mean ± standard deviation. Values with different superscripts in each row are significantly ($p < 0.05$) different from each other.

Figure 4.14 BGN probiotic beverage served in coded atcha jar for sensory evaluation

beverages and the least rated plain beverages was the sucrose and the vanilla essence. Fermentation resulted in the lowering of the pH which in turn lightens the product (Nnam, 1997).

Mean rating for aroma ranged from 1.8 (*L. bulgaricus & L. plantarum* plain) to 2.7 for *L. bulgaricus* sweetened. The vanilla essence and the volatile organic acids produced during fermentation could be attributed to the difference which was picked up by the panellist. Mean rating for taste ranged from 1.5 for *L. bulgaricus* plain to 2.9 for *L. bulgaricus* sweetened. *L. bulgaricus* sweetened was rated the highest, the sucrose and vanilla essence contributed to higher rating. During fermentation *L. bulgaricus* produces a characteristic tartness and the green flavour of yoghurt (Oliveria *et al*., 2002) which is perceived both through aroma and taste. Non-volatile acids such as lactic or pyruvic, volatile acids: butyric or acetic, carbonyl compounds: acetaldehyde or diacetyl and miscellaneous compounds: amino acids, products from thermal degradation, are natural preservatives and contribute to the perceived aroma and taste characteristics (Serra, 2009).

Mean rating for mouthfeel ranged from 1.8 (*L. bulgaricus & L. plantarum plain*) to 2.7 for *L. bulgaricus* sweetened. Mouthfeel was the BGN probiotic beverage physical and chemical interaction with the mouth. The difference perceived in mouthfeel was as a result of the strain differences and the added sucrose. The gelatinised starch aided to the perceived mouthfeel. Tannins, which are located in the seed coat, could have also contributed to the mouthfeel, to a lesser extent though. It is expected that the high temperature and pressure of sterilisation along with the initial hydration of the flour would have reduced the antinutritional factors in the seeds (Brough *et al*., 1993, Nnam, 1997 & Nti, 2009). It is not surprising that some of the panellist perceived a nutty flavour (Nnam, 1997; Nti, 2009).

Overall acceptability of the samples ranged from 1.9 (*L. bulgaricus & L. plantarum* plain) to 2.8 (*L. bulgaricus* sweetened). The *L. bulgaricus* sweetened sample was higher in overall acceptability being moderately desirable. General acceptability of the milk is influenced by the various organoleptic attributes of flavour, texture and colour.

4.3.8 Market segmentation of BGNPB based on consumer preference

The dendrogram (not shown) obtained from the hierarchical cluster analysis indicated that the panellists could be divided into two groups on the basis of probiotic beverage appearance, colour, aroma, taste, mouthfeel and overall acceptability. The sensory quality indices (appearance, colour, aroma, taste, mouthfeel and overall acceptability) were subjected to principal component analysis (PCA) to identify the two groups. The suitability of the data for factor analysis was assessed prior to performing PCA. Inspection of the correlation matrix revealed the presence of many coefficients of 0.3 and above. The Kaiser-Meyer-Oklin value (0.812) exceeded the recommended value of 0.6. The Barlett's test of sphericity was significant ($p < 0.05$). Hence, factorability of the correlation matrix is supported.

PCA revealed that the variation in the sensory characteristics of the BGN probiotic beverage could be explained by two components with eigenvalues exceeding 1. Much of the variation (47.4%) in the data was explained by component 1 and 29.3% by component 2, with a cumulative variation of 76.7% of the variation explained by the components. This was further supported by the clear break after the second component in the screeplot (not shown).

Varimax rotation was performed to aid in the interpretation of these two components (Table 4.11). Taste and mouthfeel loaded strongly on component 1, while appearance and colour on component 2. The component score coefficient matrix indicated that component 1 was mouthfeel while component 2 correlated to colour. The implication is that BGNPB can be categorised into market groups on the basis of mouthfeel, taste, appearance and colour. K-means cluster analysis was performed to characterise the groupings on the basis of these sensory attributes. Cluster 1 (44.4% cases) was the BGNPB that was moderately desirable (rated 3) in appearance, colour, taste and mouthfeel. Cluster 2 (55.6%) was those that were moderately undesirable (rated 2) in appearance and colour; very undesirable (rated 1) in mouthfeel and taste. Hence, mouthfeel taste, appearance, and colour were the important parameters considered by the panellist in their preference.

Table 4.12 indicates the cross-tabulation of the probiotic beverages samples according to their cluster membership with respect to sensory attribute and age.

Table 4.11 Pattern/structure for coefficients obtained using varimax rotation of two factor solution for sensory attributes of BGNPB

	Component	
Quality	1	2
Appearance	-0.189	0.625
Colour	-0.193	0.628
Aroma	0.255	-0.002
Taste	0.379	-0.156
Mouthfeel	0.382	-0.168
Overall acceptability	0.304	-0.042
% of variance explained	47.4	29.3

BGNPB - bambara groundnut probiotic beverage

Table 4.12 BGN probiotic beverages clusters on the basis of sensory attributes*

Character	Cluster Number of Case**		Total
	1	2	
Probiotic			
L. bulgaricus plain	9 (22.5)	31 (77.5)	40 (100)
L. bulgaricus & L. plantarum plain	7 (17.5)	33 (82.5)	40 (100)
L. bulgaricus sweetened	29 (72.5)	11 (27.5)	40 (100)
L. bulgaricus & *L. plantarum* sweetened	26 (65)	14 (35)	40 (100)
Total	83 (53.2)	73(46.8)	156 (100)
Panellists age			
<20	2 (25)	6(75)	8 (100)
20-30	38 (37.6)	63 (62.4)	101 (100)
31-40	22 (62.9)	5 (37.1)	35 (100)
>40	7(58.3)	5(41.7)	12(100)
Total	69 (44.2)	87 (55.8)	156 (100)

*Numbers are frequency and percentage in bracket.

**Cluster 1 = moderately desirable (rated 3) both in taste and mouthfeel; Cluster 2 = moderately undesirable (rated 2) in appearance and colour and very undesirable (rated1) in mouthfeel and taster.

In terms of sensory attribute, the *L. bulgaricus* sweetened (72.5% cases) and the *L. bulgaricus* & *L. plantarum* sweetened (65.0% cases) belong to cluster 1- moderately desirable in appearance, colour, taste and mouthfeel. Cluster 2 consisted of *L. bulgaricus* plain (77.5% cases) and *L. bulgaricus* & *L. plantarum* plain (82.5% cases) being moderately undesirable (rated 2) in appearance and colour and very undesirable (rated 1) in mouthfeel and taste. The majority of the cases between 31-40 (62.9%) and majority in age > 40 (58.3%) equivalent of 44.2% of the panellists are in cluster 1, indicating they moderately desired the BGNPB in appearance, taste and mouthfeel. There was a high preference for BGN probiotic beverage across all the age groups. The high preference for panellist >31 years of age indicates the high probability of acceptance of BGN probiotic beverage if commercialised. Future work will give attention to increase the sucrose thereby improving the taste. The objective of developing a probiotic beverage using BGNM was achieved; hence BGNM was a good substrate for the growth LAB.

4.4 Conclusion

Physical properties of BGNPB were affected by strain used for fermentation; BGNPB could be classified into two groups on sensory basis. The consumers moderately desired the beverage with 3 in taste and mouthfeel. Sucrose also affected consumer acceptability of BGNPB. BGNM supported the growth of probiotics. Optimal growth of *L. bulgaricus* and *L. plantarum* was estimated to 35^{o}C for 24 h. It was possible to produce BGNPB using the two stains. The consumers moderately desired the beverage in taste and mouthfeel. Taste, appearance, and colour were the important parameters considered by the panellist in their preference. Preference for BGN probiotic beverage was high among the > 40 age groups, demonstrating the potential of BGNM when more sucrose is added and commercialised.

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CHAPTER 5

PROXIMATE, ANTIOXIDANT ACTIVITY, PROBIOTIC TOLERANCE TO SIMULATED GASTRIC JUICE AND BILE AND SHELF LIFE CHARACTERISTICS OF BAMBARA GROUNDNUT PROBIOTIC BEVERAGE

ABSTRACT

Two bambara groundnut probiotic beverages (BGNPB), fermented with (1) *L. bulgaricus* and (2) *L. bulgaricus* and *L. plantarum* were assessed for their proximate composition, antioxidative activity, probiotic tolerance to simulated gastric juices and bile *in vitro* and a 28-day shelf life study at (5, 15 and 25^{o}C). AOAC methods were used in quantifying the proximate analysis of the BGNPB. Antioxidative activity was assessed using the 1, 1-diphenyl-2-picrylhy-drazyl (DPPH). The BGNPB were exposed to gastric juice (pH 2.0, 2.5 and 3.0) and bile (0.3, 0.5 and 1%) for 180 and 240 minutes, respectively. Sampling was done every 30 minutes for optical density (OD) at 600 nm. The OD was then used to determine percent bacterial survival. During the shelf life study sampling of the BGNPB was done at 2-day interval for 28 days for, pH, titratable acidity (TTA), microbial load (Log cfu/mL) and colour. The shelf life data was modelled with Mitscherlich's law of diminishing returns model and used to estimate the shelf life of the BGNPB. Protein content was 19.9 ± 0.4% and 22.3 ± 0.8%, total fat 8.0 ± 1.0% and 9.0 ± 0.9%, total dietary fibre (TDF) 43.9 ± 0.9% and 29.1 ± 1.1%, carbohydrates 23.0 ± 0.7% and 30.0 ± 4.6%; ash 4.47 ± 0.12% and 5.46 ± 0.46 and antioxidative activity of 5.01 ± 0.1% and 14.2 ± 1.0% for (1) *L. bulgaricus* and (2) *L. bulgaricus* and *L. plantarum,* BGNPB respectively. Concentrations of the gastric juice, bile and time of incubation of the BGNPB had significant ($p < 0.05$) effect on the percent survival of the probiotics. Best survival was 23.1% (3 Log reductions) after 180 min at pH 3.0 and 11.4% (4 Log reductions) for 0.3% bile after 240 min for both beverages. After the 28 day storage pH was 4.30 and 4.25 at 5^{o}C; 3.94 and 3.72 at 15^{o}C and 3.78 and 3.45 at 25^{o}C for (1) *L. bulgaricus* and

(2) *L. bulgaricus* and *L. plantarum,* respectively. TTA was 1.8% and 2.0% at 5^{o}C; 2.0% and 2.2% at 15^{o}C while at 25^{o}C it was 2.6% and 2.6% for (1) *L. bulgaricus* and (2) *L. bulgaricus* and *L. plantarum,* respectively. Microbial load after the 28 days study was 7.05 Log cfu/mL for *L. bulgaricus* and 7.11 Log cfu/mL for *L. bulgaricus* and *L. plantarum*; BGNPB at 5^{o}C. However no probiotic was detected at 15 and 25^{o}C for the two BGNPB. Lightness was 55.28 and 56.13 at 5^{o}C; 63.5 and 64.43 at 15^{o}C while 66.96 and 67.95 at 25^{o}C for (1) *L. bulgaricus* and (2) *L bulgaricus* and *L. plantarum,* respectively. Redness, yellowness, chroma and hue angle were significantly affected by the storage temperatures and time. Shelf life was estimated as 28 days (right censored) for both BGNPB stored at 5^{o}C, 18 days and 10 days, respectively for (1) *L. bulgaricus* and (2) *L. bulgaricus* and *L. plantarum* at 15^{o}C, 2 days for both at 25^{o}C. BGNPB was high in proteins, total dietary fibre with antioxidative activity and protected the probiotics against *in vitro* gastric juice and bile.

5.1 Introduction

Fermented vegetable products are nutritious foods containing high amounts of nutrients and minerals (Obizoba *et al*., 1992; Nnam 1997; Amarteifio & Moholo, 1998; Omoikhoje, 2008; Granato *et al*., 2010). Human consumption of yoghurt has been associated with tremendous health benefits due to improvements of gastrointestinal functions and disease (Granato *et al*., 2010; Olugbuyiro & Oseh, 2011). Food matrix and composition of the medium affect the survival and growth of probiotics in the product and after ingestion (Charteris *et al.,* 1998, Pinto *et al*., 2006; Mishra *et al,.* 2008). Legumes contain bioactive substances (Lam & De Lumen 2003; Berk *et al*., 2011), that protect food quality by retarding oxidative breakdown of lipids (White & Xing, 2008), the human cells and tissue from the damaging effects of toxic molecules called free radicals (Adelakun *et al.,* 2009). It is expected that bambara groundnuts (BGN) and its milk (BGNM) as reported in sections 3.3.9, page 78 contains such bioactive compounds.

In the previous work we established that BGNM could support the growth of lactic acid bacteria (section 4.3.2, page 111). We also produced consumer acceptable bambara probiotic beverage (BGNPB) as reported in section 4.3.7, page 134. Probiotics are defined as “live microorganisms which when

administered in adequate amounts confer a health benefit on the host" (Champagne & Gardner, 2008; Prado *et al.*, 2008). Two of the most genera found in human gut are *Lactobacillus* and *Bifidobacterium* (Oliveria *et al*., 2001; Singh *et al*., 2012). However, for the benefits to be experienced a sufficient amount of probiotics must reach the intestine in sufficient amounts (Schillinger, 1999; Bao *et al.,* 2010; Ng *et al.,* 2011). The viability of probiotics in the products has been cited as an important prerequisite for achieving the beneficial health effects (Schillinger, 1999; Bao *et al.,* 2010).

Poor storage conditions can lead to spoilage of yoghurt within a short period of time (Mataragas *et al*., 2011). Spoilage of yoghurt is as a result of changes in its physical, chemical, organoleptic characteristics (Kneifel *et al.,* 1993; Shimakawa *et al*., 2003) and microbial load (Ng *et al*., 2011), making it unsuitable for human consumption (Matagaras *et al.,* 2011) and diminishing the potential health benefits conferred by the products (Ng *et al*., 2011). Maintaining a high load of viable probiotics is threatened by a number of factors, strain variation, acid accumulation and acidity of the product, levels of oxygen in the products and permeability of oxygen through packaging (Oliveria *et al*., 2001; Birollo *et al*. 2002; Shimakawa *et al*., 2003). Champagne & Gardener (2008) stated that probiotic bacteria loose viability during storage in many fermented milk having pH values below 4.0. Nevertheless viability during storage and conferring health benefits are of primary importance although there are instances where non-viable cells have shown health benefits (Salminen *et al.,* 1999; Ouwehand & Salminen 1998). However, it is still desirable to have live cultures in the product, to comply to the regulated specifications. One of the largely relied tool for predicting shelf life is the use of predictive models (Mataragas *et al*., 2011).

After ingestion, probiotic bacteria must overcome two main biological barriers, the acidic environment of the stomach and bile secreted in the duodenum (Kneifel *et al.,* 1993; Schillinger, 1999; Olivera *et al*., 2001; Shimakawa *et al.,* 2003). It is not clear whether probiotics can grow in the intestinal environment. However, survival of probiotic strains under the gastrointestinal tract conditions is strongly affected by the nature of the food carrier, its buffering capacity, pH, chemical and physical characteristics (Oliveria *et al*., 2001; Pinto *et al*., 2006). Probiotics must be metabolically active during the gastrointestinal transit and must reach the colon in large numbers so as to facilitate colonization (Lian *et al.* 2003).

Two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts, based on survival and growth studies (Vinderola & Reinheimer, 2003). Probiotics naturally tolerate acid and bile. Food ingredients such as proteins, polysaccharide and free amino acids, have been reported to have a protective effect on them and enhance their survival in gastrointestinal tract (Charteris *et al*., 1998; Pinto *et al.,* 2006).

Human stomach pH ranges from 1.5 during fasting to 4.5 after a meal (Jacobsen *et al.,* 1999). About 2.5 L gastric juice at a pH of approximately 2.0 is secreted into the stomach (Charteris *et al.,* 1998) which causes the destruction of most microorganisms ingested. Relevant physiological concentration of human bile ranges from 0.3% to 0.5%, which is ideal when conducting *in vitro tests* (Dunne *et al*., 2001).

Mathara *et al*. (2008) reported that *L. fermentum* showed almost 100% survival under simulated stomach acidic conditions and physiological salt concentration of bile. To our knowledge there was no documentation on the gastric and bile tolerance of *L. bulgaricus* and *L. plantarum* in fermented BGNM. Therefore the objectives of this study were to: (1) determine the proximate and antioxidative activity of BGNPB (2) establish the shelf life of BGNPB and (3) evaluate the survival of LAB in BGNPB when exposed to simulated gastric juice and bile.

5.2 Materials and methods

5.2.1 Source of BGN, starter cultures and chemicals

Bambara groundnuts (BGN) were purchased from Thusano Products, Louis Trichardt, Limpopo, South Africa. The BGN seeds were sorted into varieties, according to different testa and hilum colours: black; red; brown; black-eye and brown-eye. Bacterial strains *L. bulgaricus* and *L. plantarum* were obtained from Anatech, Johannesburg in South Africa and Quantum Biotechnologies, Cape Town, South Africa. API 50 CH system was obtained from BioMerieux, Johannesburg, South Africa, oxgall and pepsin were obtained from Biocom biotechnology, Cape Town, South Africa.

5.2.2 Culture validation and subculturing

Confirmation of the starter culture was done following the method reported in section 4.2.4 page 100.

5.2.3 Production of BGN flour

The red BGN seeds chosen on basis of being the best to support the growth of *Lactobacillus bulgaricus*, as reported in section 3.2.2, page 51 were milled into flour using a hammer mill (Bauermeister Bauermeister Inc., Vernon Hills) with a sieve size 250 µm (No. 60), packed in clear plastic bag and stored in a refrigerator at 4 ± 2^{o}C, till needed.

5.2.4 Production of BGNM

The red variant BGNM was prepared as indicated in section 4.2 Fig. 4.1 following the method reported in the previous section 3.2.3, page 51.

5.2.5 Production of BGNPB

BGNM (100 mL x 2) in 250 mL schott bottles was warmed to 45^{o}C in a water bath. The BGNM was then inoculated with 3% of *L. bulgaricus* and another with *L. bulgaricus* and *L*. *plantarum* (1:1) and incubated in a Memmert incubator at 35^{o}C for 24 h as estimated in the previous study. The fermented BGNM designated as BGNPB was then cooled in an ice bath and stored in refrigerator at 4 ± 2^{o}C. The two samples were (1) *L. bulgaricus* BGNPB and (2) *L. bulgaricus* and *L. plantarum* BGNPB.

5.2.6 Proximate analysis of BGNPB

Protein determination of the BGNPB was done using Kjeldahl N X 6.25, total fat determined by the solvent extraction method using the Soxtex, ash and for moisture according to AOAC (2000) method 32. 1. 05 and 27.4. 4. 03. Determination of total dietary fibre was done using the Fibretec system. Total carbohydrates were obtained by difference and energy values were calculated according to Anon (2010).

5.2.7 Antioxidative activity of BGNPB

The antioxidative activity of the BGNPB was determined using the method described by Berk *et al.* (2011). The antioxidative activity of BGNPB was measured from the bleaching of purple coloured solution of 1, 1-diphenyl-2-picrylhy-drazyl (DPPH). BGNPB (3 mg mL^{-1}) in water (1 mg mL^{-1}) was added to a 3 mL of DPPH in 50% methanol (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and allowed to stand for 30 min in foil covered test tubes. Absorbance was measured at 517 nm using a UV/Vis Spectrometer (PerkinElmer Lambda 25). Antioxidative activity of BGNPB percent (AA%) was calculated using the equation:

…… Equation 5.1

where the absorbance of the control ($A_{control}$) reaction contains all the reagents expect the BGNPB.

5.2.8 Preparation of gastric juice

Simulated gastric juice was prepared according to the method described by Lian *et al*. (2003) and Huang & Adams (2004). Pepsin (1:10000, ICN) was suspended in sterile 0.5% sodium chloride to a final concentration of 3g L^{-1} and the pH adjusted to 2.0; 2.5 and 3.0 using 12 M HCl or 0.1 M NaOH. The solution was then made sterile by filtering it through a membrane (0.45 µm) and kept at 4^{o}C till needed.

5.2.9 Preparation of bile solution

Simulated bile juice was prepared according to the method described by Lian *et al.* (2003) and Vinderola & Reinheimer (2003) by first preparing oxgall solution by dissolving 10 g of oxgall in 90 mL distilled water. The oxgall solution was then used to prepare 0.3; 0.5; and 1.0% of bile, pH adjusted to 8.0 using sterile 0.1 M sodium hydroxide. Solution was sterilized at 121^{o}C for 15 minutes and kept at 4^{o}C till needed.

5.2.10 *In vitro* gastric juice and bile tolerance

The gastric juice and bile tolerance of the BGNPB were determined using the method described by Wang *et al*. (2007), with slight modification. Aliquots (2 mL) of the BGNPB were mixed with 10 mL of simulated gastric and bile juices each.

The mixture was then incubated at 37^{o}C. In gastric juice tolerance measurements, aliquots of 1 mL were taken after 0, 30, 60, 90, 120, 150 and 180 minutes. In bile tolerance measurements were taken after 0, 30, 60, 90, 120, 150, 180 and 240 min. Optical density (OD) of the mixture was measured using UV/Vis Spectrometer (PerkinElmer Lambda 25) at absorbance 600 nm and the control measured without the gastric juice/bile. Results were reported as the percentage of survival in the presence of gastric juice/ bile compared to the control

5.2.11 Shelf life stability of BGNPB

The BGNPB cold filled in 250 mL polyethylene terephthalate (PET) (rectangular 127 H x (63 x 50), mass 15 g), plastic bottles (Hilfort plastics, Cape Town, South Africa), were stored in a refrigerator at 5^{o}C, while the other two sets were stored in two Memmert incubators at 15^{o}C and 25^{o}C. The samples were monitored for pH, titratable acidity, microbial load and colour every two days for 28 days. The methodologies are described below.

pH of BGNPB during storage

The pH of the BGNM (20 mL) was measured at room temperature (21 ± 2^{o}C) using a Knick pH-Meter 766 meter, Elscolab, Nederland.

Titratable acidity of BGNPB during storage

Titratable acidity (TTA) was determined according to the South African Legislation on dairy products (Anon, 1997). Fermented BGNM (9 ml) was pipetted into an Erlenmeyer flask (250 mL). To it 0.5 mL of a 1.6% phenolphthalein indicator solution in 50% ethanol were added. The fermented BGNM was then titrated against 0.1 M NaOH solution until the first tinge of pink that persists for 30 seconds appeared. To express the titratable acidity of the BGNPB as the percentage of lactic acid, the number of millilitres of 0.1 M NaOH used in the test was divided by 10.

$$\frac{\qquad\qquad}{\qquad\qquad} \qquad \text{.....Equation 5.2}$$

Microbial load of BGNPB during storage

The probiotic bacterial load was determined according to the method described by Wang *et al.* (2007). BGNPB, (1) *L. bulgaricus* plain and (2) *L. bulgaricus* & *L.*

plantarum plain, (1.0 mL each) were mixed with 9.0 mL of 0.1% sterile peptone solution, serial dilution up to nine were made. Aliquot (1.0 mL) of dilutions (3, 6 & 9) were plated out on 90 mm Petri dishes and poured MRS agar (12 ± 2 mL) (Anatech, South Africa). When the plates had set they were double layered with MRS agar (9 ± 2 mL), placed in an anaerobic jar and incubated at 37^{o}C for 48 h.

Colour of BGNPB during storage

The colour of the BGNMP (50 mL) samples was measured using a Hunterlab Colorflex Spectrophotometer 45^{o}/0^{o} standard, set at standard observer 10^{o} and D65 and standardised with white tile (L* = 93.41, a* = -1.18, b* = 0.75) . The BGNPB samples were placed in glass samples holder and reflectance measured for L*a*b* and L*C*h colour scales. L* is lightness with 100 as maximum indicating a perfect reflecting diffuser and the minimum is zero, which is black. The a* and b* axes have no specific numerical limits, negative a* is green, positive is red. Negative b* is blue, positive b* is yellow (Aidoo *et al*., 2010). Chroma (C) is the quality that distinguishes a pure hue from a grey shade and describes hue saturation or purity; its axis extends from the values (lightness) axis towards the pure hue (Sahin & Samnu, 2006).

5.2.12 Data analysis

All results were reported as mean of three independent trials. Analysis of variance (ANOVA) was used to determine the differences between treatments. Duncan's multiple range tests was used to separate means where differences existed (IBM SSPS, 2010).

To estimate the shelf life of BGNPB the Mitscherlich's law of diminishing returns model was used to model the effect of storage time on the pH of the BGNPB. In this model b_1 is the lower asymptote; b_2 is the difference between the maximum pH and the lower asymptote and b_3 is the rate at which the minimum pH is reached known as the rate constant (day^{-1}). The model parameters were estimated using the non-linear regression model following the sequential quadratic programming estimation method. The coefficient of determination (R^2) for each regression equation, a measure of the variability in the dependent variable accounted by the model was calculated as (IBM SPSS, 2010):

…… Equation 5.3

The effect of temperature on the beverage was modelled with Arrhenius equation (Jideani *et al*., 2002).

…… Equation 5.4

where = reaction rate constant; = quality rate constant at reference temperature ; = absolute temperature (in degrees K); =.apparent activation energy (in J/mol); = universal gas constant (8.314 kJ/mol K). The reference temperature (288 K or 15^{o}C) was the mean of the value of the temperature studied.

5.3 Results and discussions

5.3.1 Proximate composition of the BGNPB

Table 5.1 details the proximate composition of the BGNPB. The protein contents were 19.8% and 22.2% for *L. bulgaricus* BGNPB and *L bulgaricus* and *L. plantarum* BGNPB, respectively. The protein content were significantly (p = 0.008 different. The difference observed can be attributed to the metabolism patterns of the individual strains used to ferment the BGNM. Nnam (1997) reported a drop in the protein content after fermentation of vegetable milk made from yam bean and mealie meal, which the author attributed to change in total nitrogen because of the assimilation of proteins by fermenting bacteria. The protein content of the BGNPB was within the range of protein content reported for BGN seeds (Apata & Ologhobo, 1994; Igbedioh *et al.,* 1994; Ijarotimi & Esho 2009; Mahala & Mohammed 2010). The protein range was almost similar to that of other documented legumes chickpea flour 23.5% and lentil flour 24.8% (Zare *et al.,* 2012).

Total fat ranged between 8 and 9% with *L. bulgaricus* and *L. plantarum* BGNPB highest. Fat content did not differ significantly (p = 0.408) between the BGNPB. The fat content range was within the documented range for BGN. Total dietary fibre was impressively high 43.9% and 29% for *L. bulgaricus* BGNPB and

Table 5.1 Proximate composition and antioxidative activity of the BGNPB[1,2]

	Bambara groundnut probiotic beverage	
Nutrient (g/100 g, d.b)	*L. bulgaricus*	*L. bulgaricus* & *L. plantarum*
Proteins	19.9 ± 0.4[a]	22.3 ± 0.8[b]
Total fat	8.0 ± 1.0[a]	9.0 ± 0.9[a]
Total dietary fibre	43.9 ± 0.9[a]	29.1 ± 1.0[b]
Carbohydrates	23.0 ± 0.7[a]	30.03 ± 4.6[a]
Ash	4.37 ± 0.12[a]	5.46 ± 0.55[b]
DPPH* (%)	5.0 ± 0.1[a]	4.2 ± 1.0[b]

[1]BGNPB – Bambara groundnut probiotic beverage; DPPH = 1, 1-diphenyl-2-picrylhy-drazyl

[2]Value are means ± standard deviation. Values with different superscripts in the same row are significantly different ($p < 0.05$).

L. bulgaricus and *L. plantarum* BGNPB, respectively. The BGNPB differed significantly ($p < 0.05$) in total dietary fibre. The high dietary fibre is of note despite sieving the BGN juice using a fine muslin cloth and discarding most solids during the BGNM production step. The high total dietary fibre makes the BGNPB an ideal substrate for probiotics and a rich source of fibre for humans,

The BGNPB ash content was 4.4% and 4.1% for *L. bulgaricus* and *L. bulgaricus* and *L. plantarum* BGNPB, respectively. Significant difference ($p = 0.016$) existed between the beverage in ash basis. A slight increase above the documented values was noted. This increase may be attributed to the breaking down of components which would have been bonding the minerals (Nnam, 1997; Mahala and Mohammed, 2010). Hence, BGNPB contains some essential minerals and vitamins needed for the body for proper functioning (Jayeola & Omueti, 2011). High mineral content as documented by Bensmira and Jiang (2012) can have some positive effect on bacterial growth and survival.

The bulk of the remaining solids were assumed to constitute the carbohydrates (Zare *et al*., 2012), which did not differ significantly ($p = 0.059$). The carbohydrates were 23% and 30% for *L. bulgaricus* and *L. bulgaricus* and *L. plantarum* BGNPB, respectively. However, the content was slightly less compared to 50-60% documented for the BGN seeds. The probiotics could have assimilated the carbohydrate resulting in the observed decrease compared to the BGN. It can be further concluded that the BGNM contains some prebiotics which the probiotics fed on, rendering the milk a good substrate for the probiotics. Zare *et al*. (2012) reported that some pulse ingredients may have beneficial effects on probiotic and yoghurt starter cultures. The result proved that BGNM could support the growth and survival of probiotics. The objective of quantifying the chemical composition of the BGNPB was achieved, and further supporting the work of other authors who described the BGN as a complete food (Mahala & Mohammed, 2010)

5.3.2 Antioxidative activity of the BGNPB

The antioxidative activity of the BGNPB is included in Table 5.1. The antioxidative activity of the two BGNPB was 5.1% for *L. bulgaricus* and 4.15% for *L. bulgaricus* and *L. plantarum*. The antioxidant activities differed significantly ($p = 0.001$). The BGNM showed relatively high antioxidative activity, the probiotics may have utilised the antioxidants.

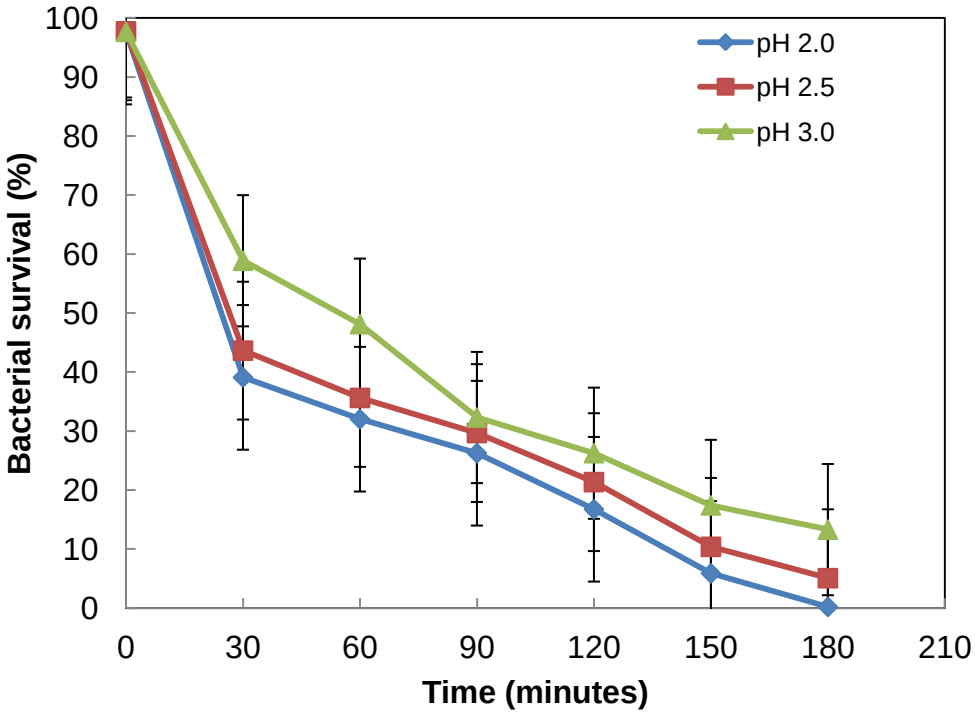
5.3.3 *In vitro* gastric juice tolerance of the BGNPB

The BGNPB with *L. bulgaricus* had a percentage bacterial survival drop from an average of 97.7% to 0.2% in pH 2.0, 5.1% in pH 2.5 and 13.3% in pH 3.0 after 180 min. While the one with *L. bulgaricus* and *L. plantarum* dropped from an average of 97% to 14.2% in pH 2.0, to 25.1% in pH 2.5 and 32.9% in pH 3.0 after 180 min. Incubation time, gastric juice pH and their interaction (time*concentration) had a significant ($p < 0.05$) effect on the survival of the probiotics in BGNPB. Survival of the probiotics decreased with incubation time and with decrease in gastric juice pH (Figure 5.1). The survival of probiotics in the two BGNPB was significantly ($F(9039.629, 19.573) = 461.832$; $p = 0.0001$) different.

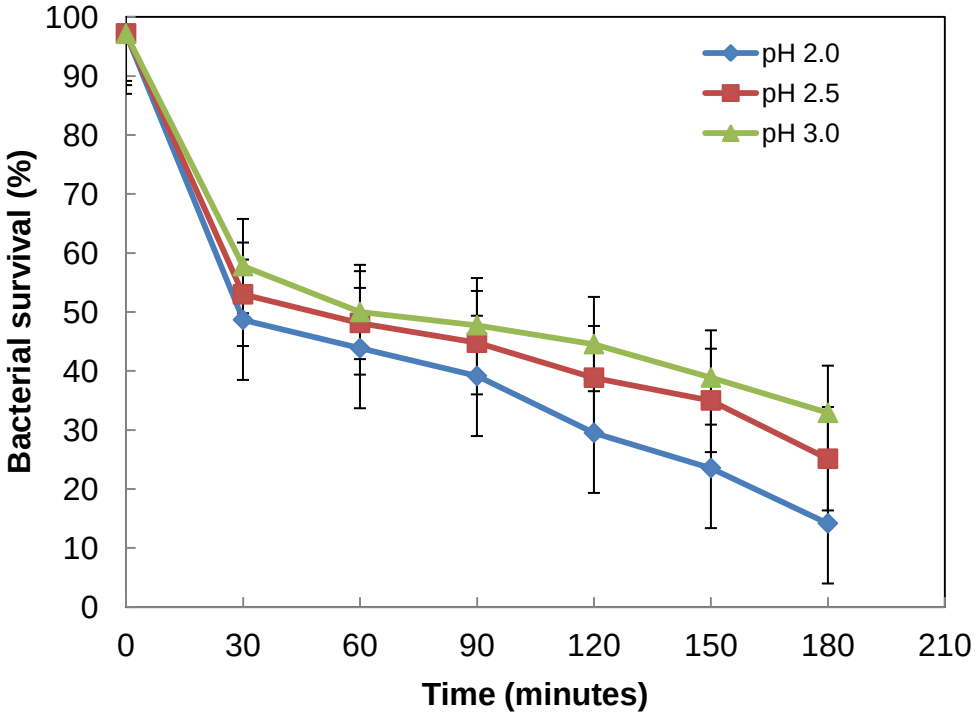
Survival in pH 3 was most promising with 13.3 and 32.9% (4 and 3 Log cfu reduction) for *L. bulgaricus* and *L. bulgaricus* and *L. plantarum* respectively after 180 min of incubation these results were in agreements with those of Goldin *et al.*, 1992 and Mishra *et al*., 2008. Survival at pH 3 is significant as ingestion with food or dairy products raises the pH in the stomach to 3.0 or higher (Martin *et al*., 1987). Charteris *et al*. (1998) reported almost complete loss of viability for *L. casei* 212.3 and F19 strains and *Lactobacillus* GG at pH 2.5 after 3 h. In this study survival was observed at pH 2.0 and 2.5. Laprisi *et al.* (2010) made similar observation, however a different observation was made by Jacobson et al (1999) where there was no survival or replication at pH 2.5. These differences can be explained by strain variability in tolerance to gastric juice (Hernandez-Hernandez *et al*., 2012).

Probiotics naturally tolerate acid and bile. Food and food ingredients such as proteins, polysaccharide and free amino acids, have been reported to have a protective effect on them and enhance their survival in gastrointestinal tract (Chateris *et al*., 1998; Pinto *et al.,* 2006). BGNPB has relatively high amounts of proteins (19-22.3%) which could have been protective to the probiotics. In the study by Pinto *et al.* (2006) the difference in survival was attributed to the different mediums used as the probiotic vehicles. Acid tolerance was much better in milk than in fruit juice due to additional protective effect of milk beside its buffering capacity in the study by Saarela *et al*. (2006).

Gastric emptying is strongly influenced by gastric contents and type of medium. Time of emptying is approximately 1.2 hours (Charteris *et al*., 1998), implying that physiologically the surviving bacteria, can reach the large intestine.



(a)



(b)

Figure 5.1 Effect of time and concentration of gastric juice on bacterial survival (a) *L. bulgaricus* and (b) *L. bulgaricus* and *L. plantarum* BGNPB

According to Charteris *et al*. (1998) 30% would be considered intrinsically tolerance to gastric juice. In this study 32.9% bacterial survival was observed for BGNPB with *L. bulgaricus* and *L plantarum* after 180 min, hence we conclude that the probiotics in the BGNPB were tolerant to gastric juice.

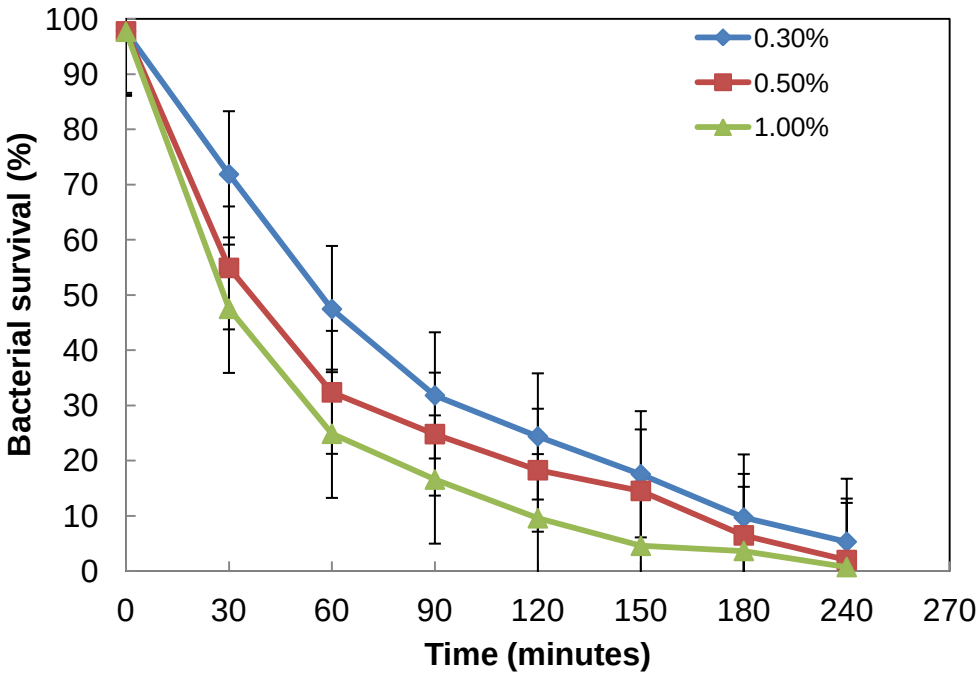
5.3.4 *In vitro* bile tolerance of the BGNPB

BGNPB with *L. bulgaricus* had a bacterial drop from 97.7% to 5.3% in 0.3% bile, 2.0% in 0.5% bile and 0.7% in 1.0% bile, while *L. bulgaricus* and *L. plantarum* dropped from an average of 97.2% to 17.5% in 0.4% bile, 12.4% in 0.5% bile and 6.1% in 1.0% bile. Time, concentration and the interaction (time*concentration) had a significant ($p < 0.05$) effect on the survival of the probiotics. The beverages had significant ($F(341833.57, 21.468)=98.124$; $p = 0.00001$) effect on probiotic survival in the simulated bile. This observation was in agreement to those of Schillinger *et al*. (2005); Bao *et al.* (2010) and Hernandez-Hernandez *et al*. (2012), who stated that the survival of probiotics in bile tolerance was strain dependent.

Survival of the probiotics decreased with time and bile concentration for each beverage (Figure 5.2). The increase in time also increased the exposure time to the bile, thus reducing the survival ability of the probiotics. Being protein in nature the probiotics could have been denatured by the bile losing their viability. Shima *et al.* (2009) reported that bile acids affect the viability of lactic acid bacteria. Vinderola and Reinheimer (2003) reported that *L. acidophilus* showed high values of resistance to gastric and bile, *L. bulgaricus* showed best probiotic characteristics among the starter species accessed. In the bile tolerance, soy proteins could bind bile acids and aggregate them firmly then protect the live bacterium from bile acids (Pinto *et al*., 2006) this could be true for the BGNPB. The objective of evaluating the tolerance of probiotics cultures in simulated gastric juice and bile BGNM was achieved; it was shown that the probiotics cultured in BGNM could survive in simulated bile.

5.3.5 Effect of storage time and temperature on pH, TTA and microbial growth of BGNPB during storage

The pH of the BGNPB with *L. bulgaricus* ranged from 4.39 to 4.3, 4.39 to 3.94 and 4.39 to 3.64 at 5, 15 and 25^{o}C storage temperatures respectively (Figure 5.3).



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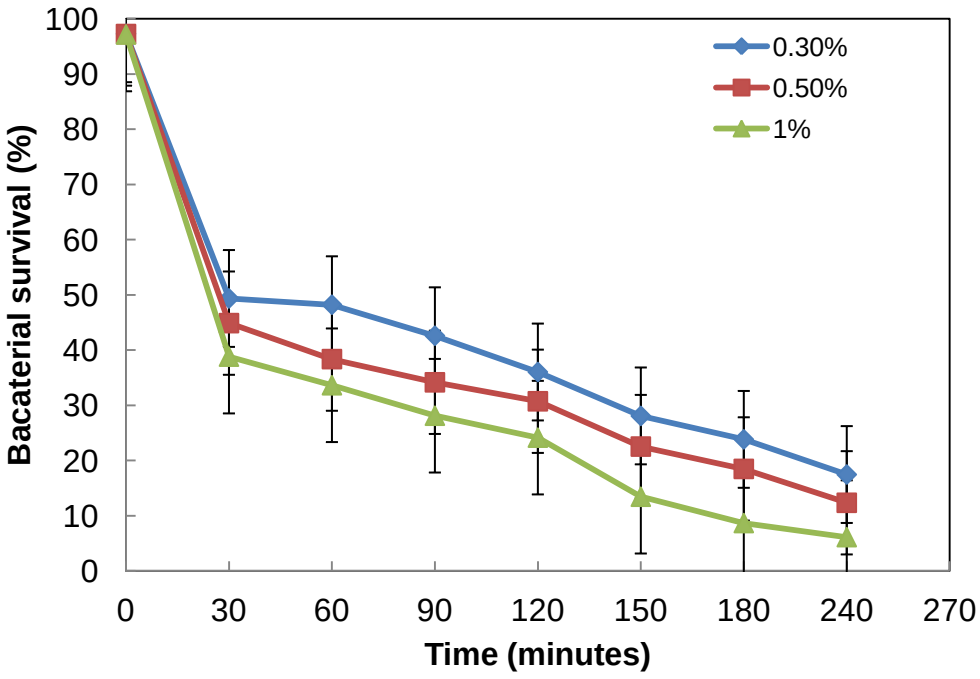
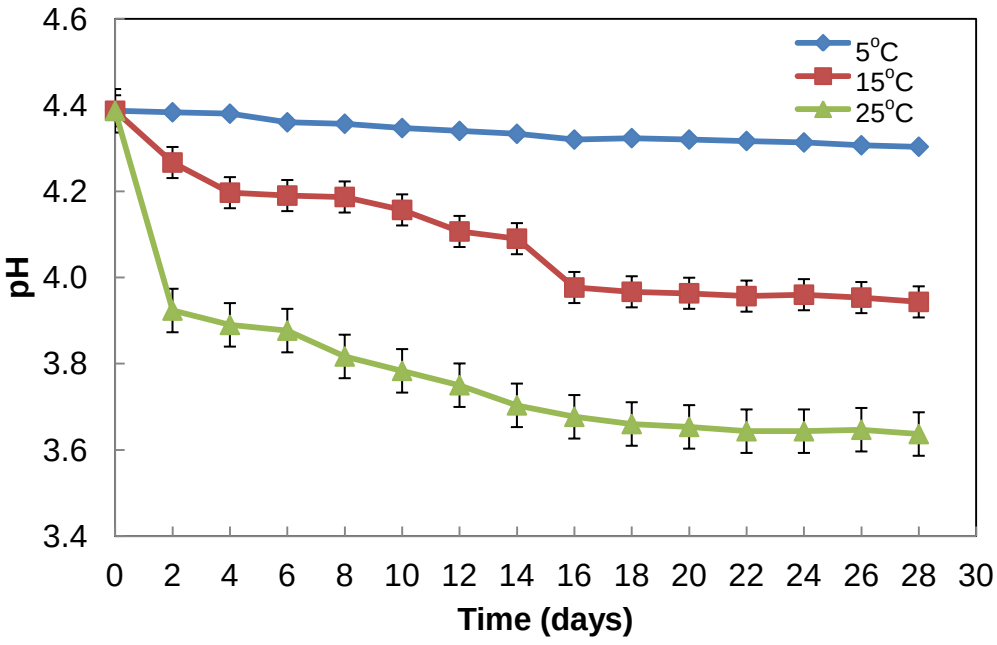


(b)

Figure 5.2 Effect of time and concentrations of bile on the bacterial survival in (a) *L. bulgaricus* and (b) *L. bulgaricus* and *L. plantarum* BGNPB.



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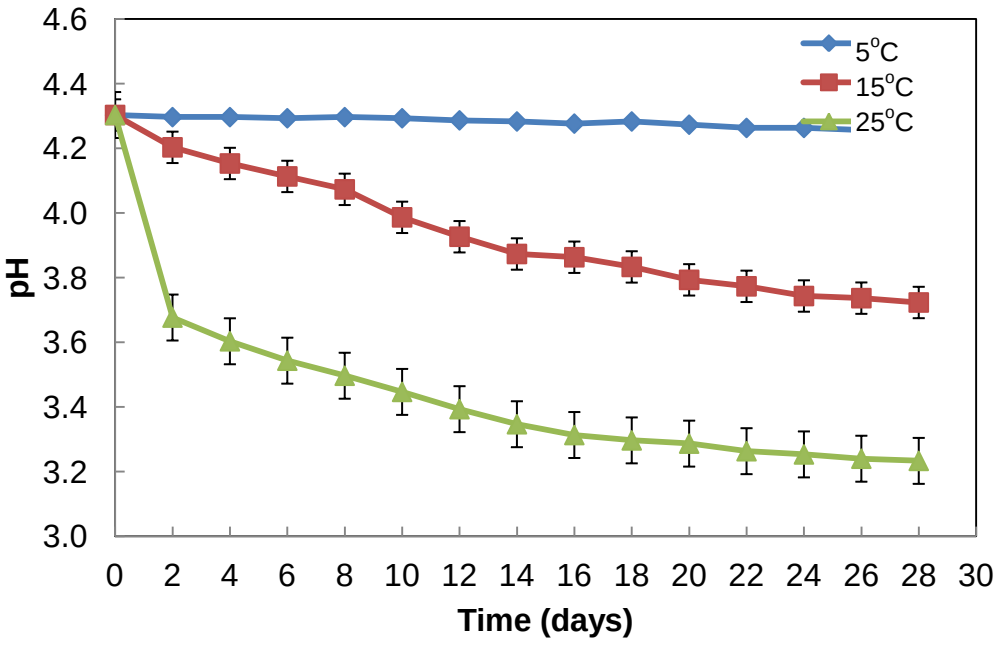
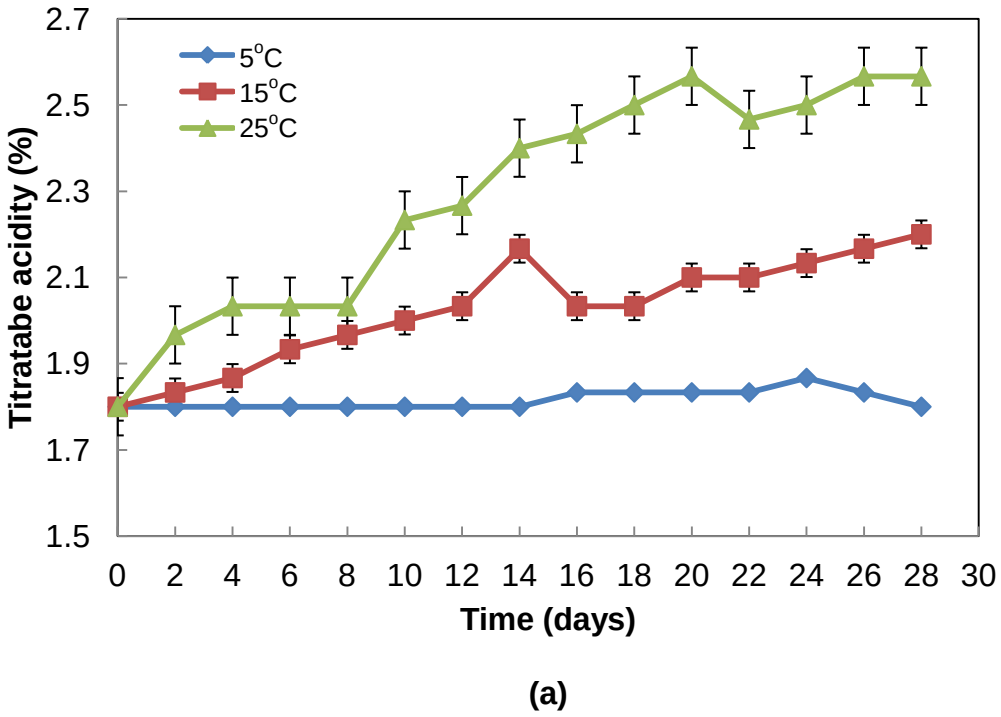


(b)

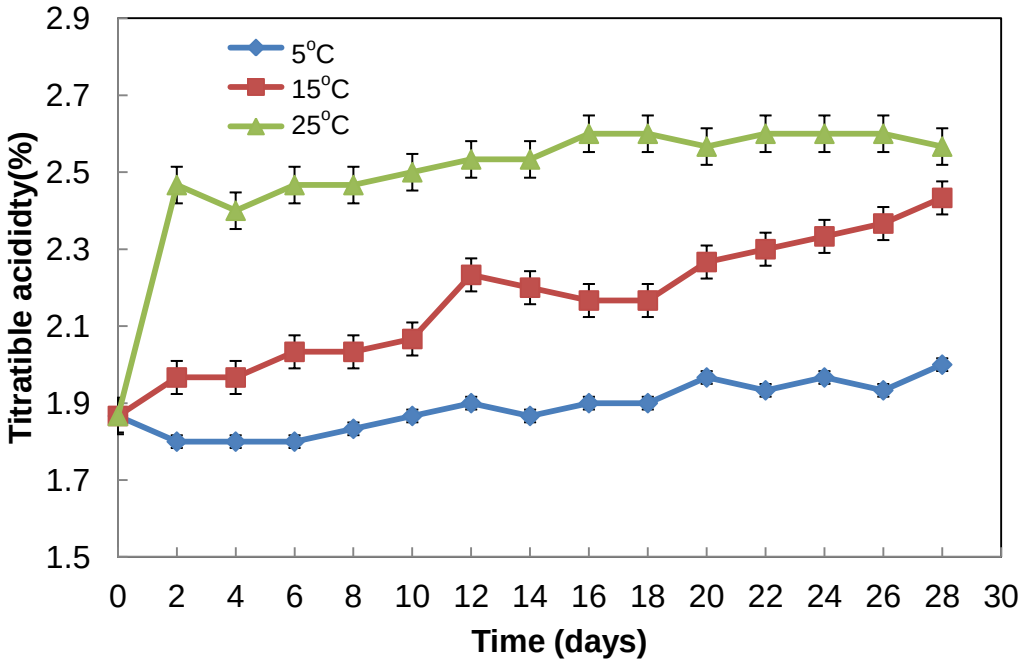
Figure 5.3 Effect of storage time and temperature on the pH of (a) *L. bulgaricus* and (b) *L. bulgaricus* and *L. plantarum* BGNPB.

While the beverage with *L. bulgaricus* and *L. plantarum* pH ranged from 4.30 to 4.25, 4.30 to 3.75 and 4.30 to 3.23 at 5, 15 and 25^{o}C storage temperatures respectively. The pH of the two BGNPB were significantly different (F (2.171, 0.005) = 434.585; p = 0.0001) from each other. The storage temperature and time had a significant (F(11.018, 0.05) = 2205.782; p = 0.0001); (F(0.334, 0.05) = 66.829; p = 0.0001) effect, respectively on the pH of the BGNPB.

Interaction of the main effect (temperature*time) had a significant (F(0.080, 0.05) = 16.046; p = 0.00001) effect on the pH of the BGNPB. BGNPB stored at 5^{o}C had a fairly stable pH. However storage at 15^{o}C and 25^{o}C resulted in a significant decrease in pH over time. The same trend was observed by Bensmira and Jiang (2011) and explained as post acidification by Ng *et al.* (2011). The pH of the BGNPB at 5^{o}C was within 4.3 to 4.2, these values were within the pH of yoghurt as documented by Tamime and Robinson (1999). The pH range was suitable for restraining pathogenic growth (Yoon *et al*., 2004; Bilgidi *et al*., 2006).
The titratable acidity (TTA) of the BGNPB with *L. bulgaricus* ranged from 1.8 to 1.87%, 1.8 to 2.25 and 1.8 to 2.57% at 5, 15 and 25^{o}C storage temperatures respectively. The beverage with *L. bulgaricus* and *L. plantarum* ranged from: 1.87% to 2%, 1.87% to 2.4% and 1.9% to 2.6%, respectively at 5, 15 and 25^{o}C. The TTA of the two beverages was significantly (F(1.254,0.007) = 189.433; p = 0.0001) affected during the period of study. The storage temperature, storage time, and their interaction significantly (F(6.560, 0.07) = 991.035; p = 0.001); (F(0.280, 0.007) = 42.250; p = 0.0001) and (F(0.056, 0.007) = 8.47; p = 0.00001) respectively, affected the TTA of the BGNPB, respectively. TTA for the BGNPB at the 5^{o}C, increased gradually, while a faster increase was noted for the BGNPB stored at 15 and 25^{o}C (Figure 5.4). TTA increased with time and was coupled with a drop in pH. Similar trends were observed by Bensmira and Jiang (2011) and Mataragas *et al*. (2011). Growth rate of microbes is linked to acidification and depended on the culture used (Zare *et al*., 2012). The trend could be attributed to the rate of reaction which was relatively higher at the higher storage temperatures. Bensmira and Jiang (2011) stated that lactic acid was the common end product of bacterial fermentation, thus the increasing TTA with storage temperature and time could have been as a result of post acidification or further growth of the bacteria involved.



(a)



(b)

Figure 5.4 Effect of storage time and temperature on the TTA of (a) *L. bulgaricus* and (b) *L. bulgaricus* and *L. plantarum* BGNPB.

BGNPB with *L. bulgaricus* had the following range for the microbial load 7.05 to 7.88 Log cfu/mL, 7.13 to 7.59 Log cfu/mL and 7.04 to 7.59 Log cfu/mL at 5, 15 and 25^{o}C storage temperatures, respectively (Figure 5.5). While the BGNPB with *L. bulgaricus* and *L. plantarum* ranged from 7.06 to 7.52 Log cfu/mL, 7.08 to 7.52 log cfu and 7 to 7.52 Log cfu/mL at 5, 15 and 25^{o}C storage temperatures. Storage temperature, time and the interaction of the main effect (temperature * time) had a significant effect on the microbial load ($F(0.930, 0.004) = 212.851$; $p = 0.0001$), ($F(1.191, 0.004) = 272.542$; $p = 0.0001$) ($F(0.244, 0.004) = 55.902$; $p = 0.0001$) ($F(0.098, 0.004) = 22.422$; $p = 0.0001$), respectively. Microbial load decreased gradually for the BGNPB stored at 5^{o}C, while the ones kept at 15 and 25^{o}C showed a sudden decrease as illustrated in Figure 5.5 during the storage period. Similar trends were reported by Ng et al. (2012) where the microbial load was decreasing with storage temperature and time.

5.3.6 Effect of storage temperature and time on the colour of BGNPB

The lightness of the BGNPB with *L. bulgaricus* ranged from 43.2 to 55.3, 43.2 to 63.5 and 43.2 to 67.0, respectively at 5, 15 and 25^{o}C storage temperature. The *L. bulgaricus* and *L plantarum* ranged from 43.4 to 56.2, 43.4 to 64.4 and 43.4 to 68.0 at 5, 15 and 25^{o}C, respectively storage temperatures. Temperature and time had significant ($F(2499, 0.773) = 3233.2$; $p = 0.0001$) and ($F(581.5, 0.773) = 752.2$; $p = 0.0001$) effects on the lightness of the BGNPB. The interaction of time and temperature also had a significant ($F(28.9, 0.773) = 37.4$; $p = 0.0001$) effect on lightness of the BGNPB. The two BGNPB were also significantly different in lightness. There was an increase in lightness with time. The increase was relatively gradual for the BGNPB stored at 5^{o}C, while a faster increase was observed for BGNPB at 15^{o}C and 25^{o}C, as illustrated in Figure 5.6. The increase in lightness was in agreement with the observation of Aidoo *et al.* (2010). The implication is that the BGNPB were becoming lighter with time. There was a negative relationship between pH and lightness of the BGNPB, decrease in pH resulted in increased lightness. Similar trend was reported by Allen *et al*. (1997).

Redness, yellowness, and hue angle of the BGNPB, were significantly ($p < 0.05$) affected by storage temperature, time and the interaction (temperature*time). Redness had a mean range of 4.2 to 6.4; yellowness 7.2 to 13.6, and hue angle 45.9 to 58.7. The redness (a*) and yellowness (b*) of the

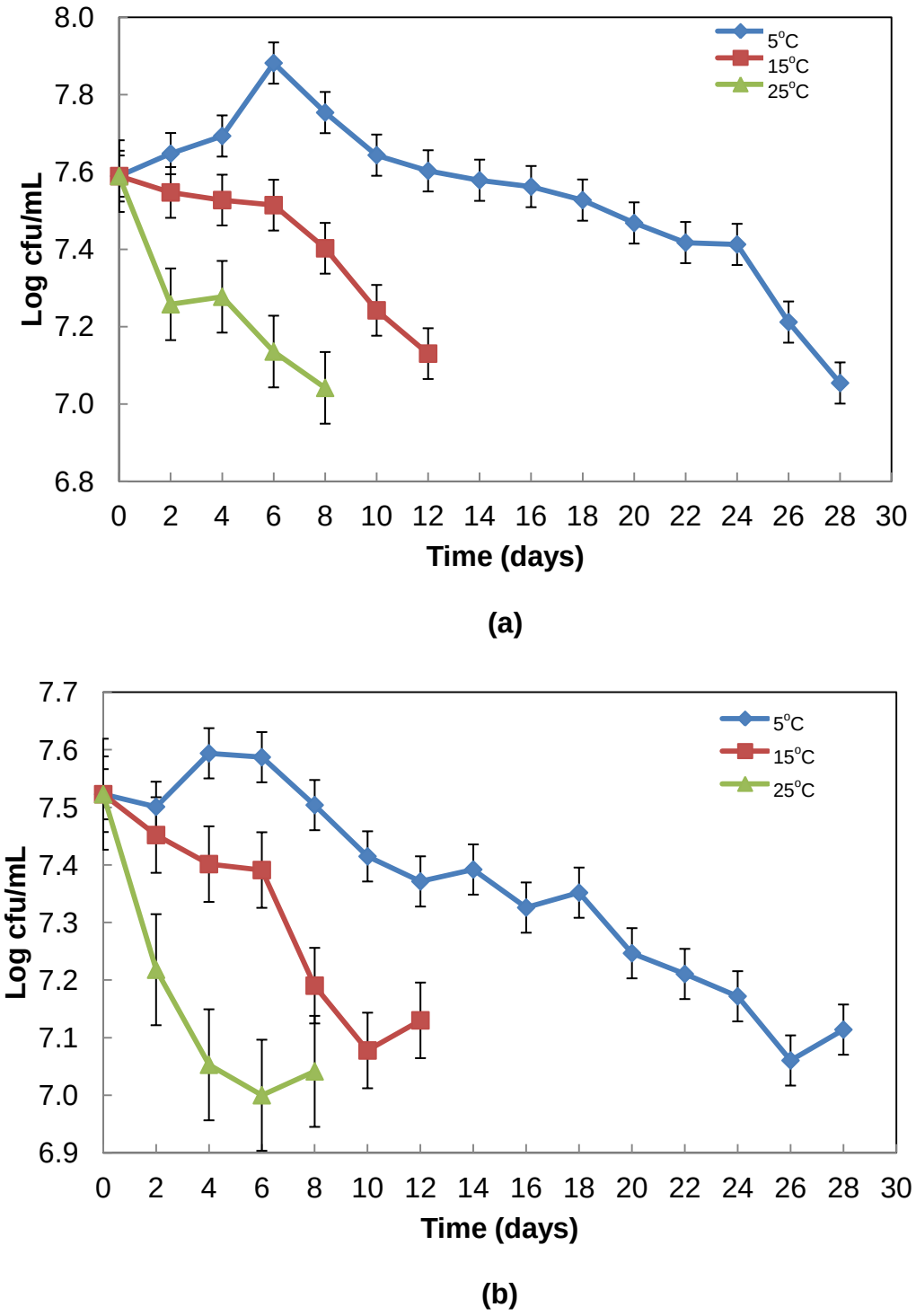
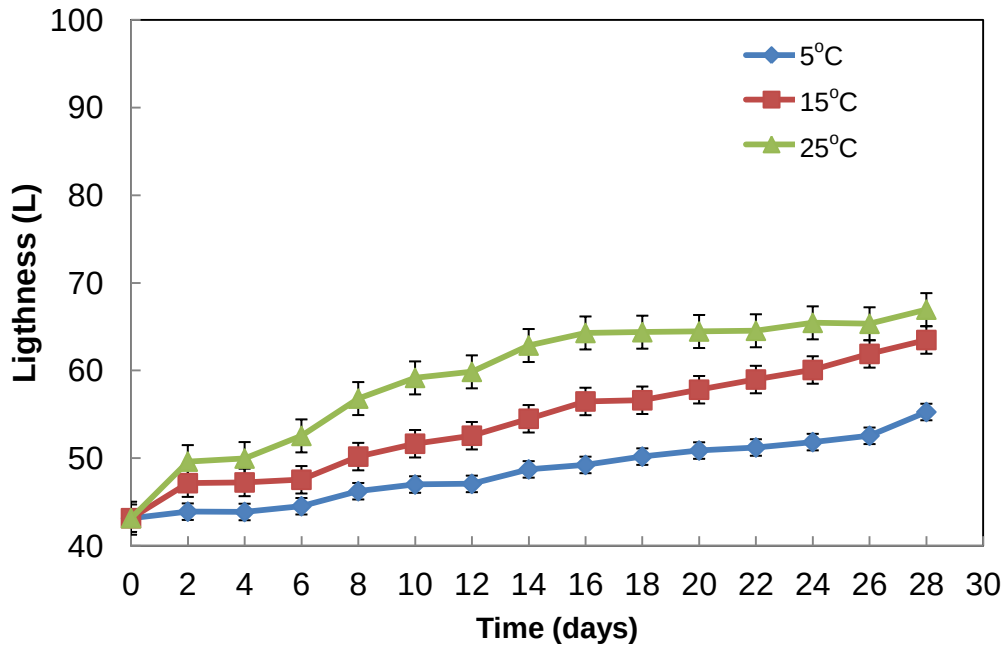


Figure 5.5 Effect of time and temperature on the microbial load of (a) *L. bulgaricus* and (b) *L. bulgaricus* and *L. plantarum* BGNPB.



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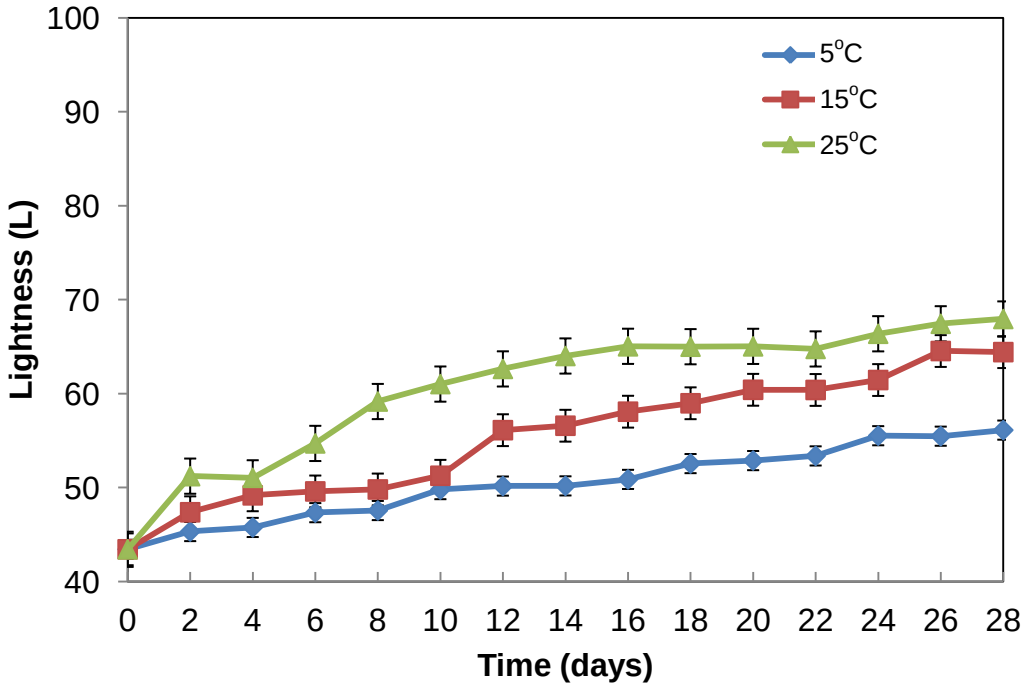


(b)

Figure 5.6 Effect of time and temperature on the lightness (L) of (a) *L. bulgaricus* and (b) *L. bulgaricus* and *L. plantarum* BGNPB**.**

BGNPB were positive which implied that the beverages are in the red and yellow colour space. This was further confirmed by the hue angles which were within the zero and 90^{o} colour space, which covers the red and the yellow space (Bible & Singha, 1993).

Chroma was not significantly affected by the temperature and time during the shelf life study. A range of 6.3 to 11.3 was observed for the BGNPB. The two BGNPB were not significantly difference in terms of chroma. The hue of the BGNPB was less saturated.

5.3.7 Modelling of shelf life of BGNPB using pH as index of deterioration

Mitscherlich's law of diminishing returns model was used to model the effect of storage time on the pH of the probiotic beverage. Table 5.2 indicates the parameters of the model for *L. bulgaricus* BGNPB and *L. bulgaricus* and *L. plantarum* BGNPB. Index of deterioration below which the probiotics may not survive in the BGNPB was chosen as pH 4. The lower asymptote (b_1) decreased with increase in storage temperature reaching below 4 at the end of the storage period in both beverages. The difference between the maximum pH and the lower asymptote (b_2) in both beverages increased with increase in temperature. The rate at which the minimum pH is reached or the rate constant (b_3) decreased as the storage temperature increased in both beverages. Rate of deterioration appeared to be faster for *L. bulgaricus* at 5 and 15^{o}C compared to that of *L. bulgaricus* and *L. plantarum*. These parameters were inserted into the Mitscherlich's law of diminishing returns model to estimate the time it will take the product to reach pH 4. The estimated shelf life is indicated in Table 5.2.

Censoring in shelf life data is the impossibility of systematically observing the failure times for all samples (Guilletand & Rodrigue, 2009). Right-censoring is the type that occurs whenever the duration of the study is fixed as in this study such that the failure time is unobservable at the end of the study period. The shelf life study was terminated after 28 days. The shelf life decreased with increase in temperature. At refrigeration temperature the beverages lasted for 28 days, right-censored value. The shelf life was estimated to be 18 days for *L. bulgaricus* BGNPB and 10 days for *L. bulgaricus* and *L. plantarum* BGNPB at 15^{o}C. For both beverages shelf life at 25^{o}C was estimated to be two days. Hence, it is imperative

Table 5.2 Parameters of the Mitscherlich's law of diminishing returns model for pH of BGNPB[1,2]

Temperature (^{o}C)	b_1	b_2	b_3 (day^{-1})	R^2	Estimated shelf life (days)
L. bulgaricus					
5	4.271 ± 0.017	0.133 ± 0.014	0.093 ± 0.023	0.93	28*
15	3.836 ± 0.042	0.591 ± 0.032	0.125 ± 0.021	0.94	18
25	3.664 ± 0.013	1.040 ± 0.078	0.470 ± 0.048	0.92	2
L. bulgaricus & L. plantarum					
5	3.374 ± 7.66	0.937 ± 7.66	0.004 ± 0.034	0.85	28*
15	3.543 ± 0.028	0.846 ±0.022	0.108 ± 0.008	0.99	10
25	3.288 ± 0.015	1.525 ± 0.103	0.497 ± 0.045	0.94	2

[1]Values ± standard error, BGNPB = Bambara groundnut probiotic beverage

[2] where b_1 = lower asymptote; b_2 = difference between the maximum pH and the lower asymptote and b_3 = rate at which the minimum pH is reached known as the rate constant (day^{-1}).

*Right-censored value – failure time was not observed at the end of the shelf life.

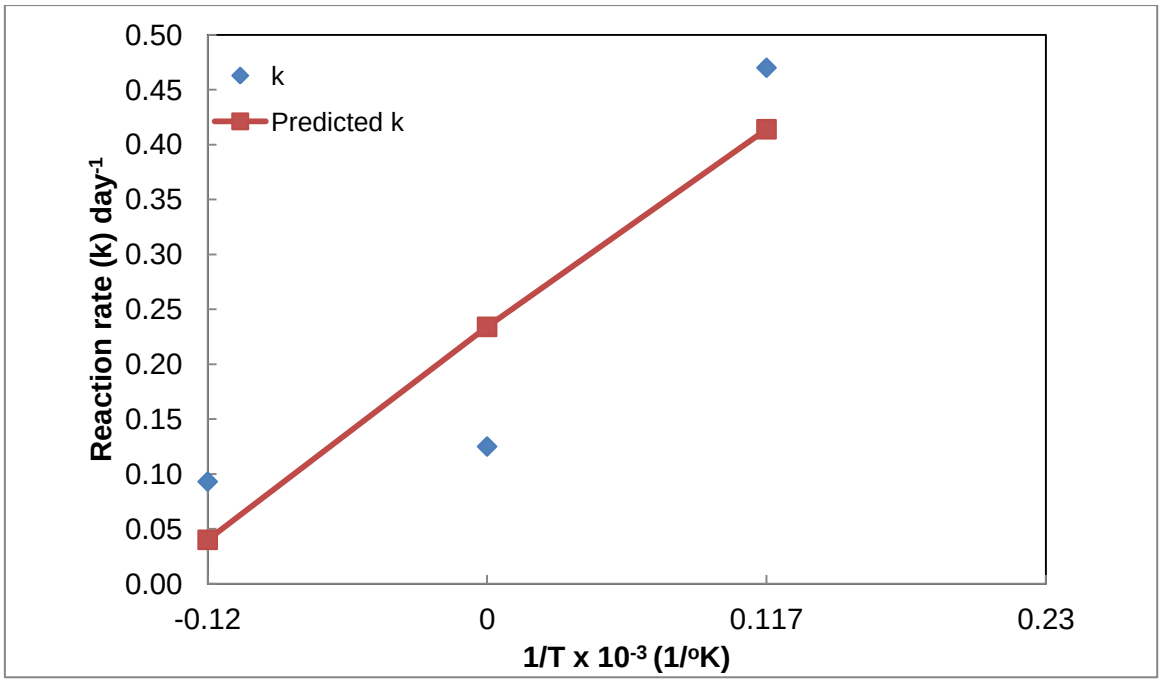
that BGNPB must be stored at refrigeration temperature in order to maximise shelf life.

5.3.8 Effect of temperature on the rate constant of BGNPB during storage

The natural logarithm of the rate constants (b_3 in Table 5.2) were regressed against the inverse of the temperature using linear regression in order to estimate the model parameters of the Arrhenius equation (Figure 5.7). Table 5.3 details the estimated parameters, the activation energy (E_a), the quality rate constant at reference temperature (k_{qref}) and the coefficient of determination (R^2). The high values of R^2 (0.80-0.89), indicates that the effect of temperature on the rate constants during storage could be described by the Arrhenius equation. Activation energy (E_a) of 12.8 kJ/mol was estimated for BGNPB with *L. bulgaricus*, while a higher value of 16.9 kJ/mol was obtained for the BGNPB with *L. bulgaricus* and *L. plantarum*. The lower activation energy for *L. bulgaricus* BGNPB further confirms that its rate of reaction was faster. The activation energy of 106 kJ/mol for lactic acid bacteria was reported by Vaikousi *et al*. (2009). The values obtained in this study suggest that the pH drop was not only attributed by the lactic acid bacteria. The difference in activation energy between the two beverages could have been caused by the different strains in each beverage.

5.4 Conclusion

The BGNPB are considered nutritious, supplying an average of 11.5% of the recommended daily allowance for proteins. The *in vitro* results suggest that the probiotic in the beverage can successfully transit the human stomach and capable of reaching the colon in relative effective numbers. Using changes in pH as the index of quality, kinetics of pH was successfully modelled using Mitscherlich's law of diminishing returns and Arrhenius equation. Kinetics studies showed that pH of BGNPB was relative stable under refrigeration (5^oC), resulting in a right censored shelf life of 28 day, 18 and 10 respectively for L. *bulgaricus* and *L .bulgaricus* and *L. plantarum* BGNPB under abused temperature (15^oC) and 2 days at accelerated storage (25^oC) for both beverages. The shelf life of the BGNPB under refrigeration (5^oC) was high when compared to their dairy counterparts with shelf life of 14 days at 4^oC. Hence, BGNPB can be a vehicle to transport probiotics to humans.



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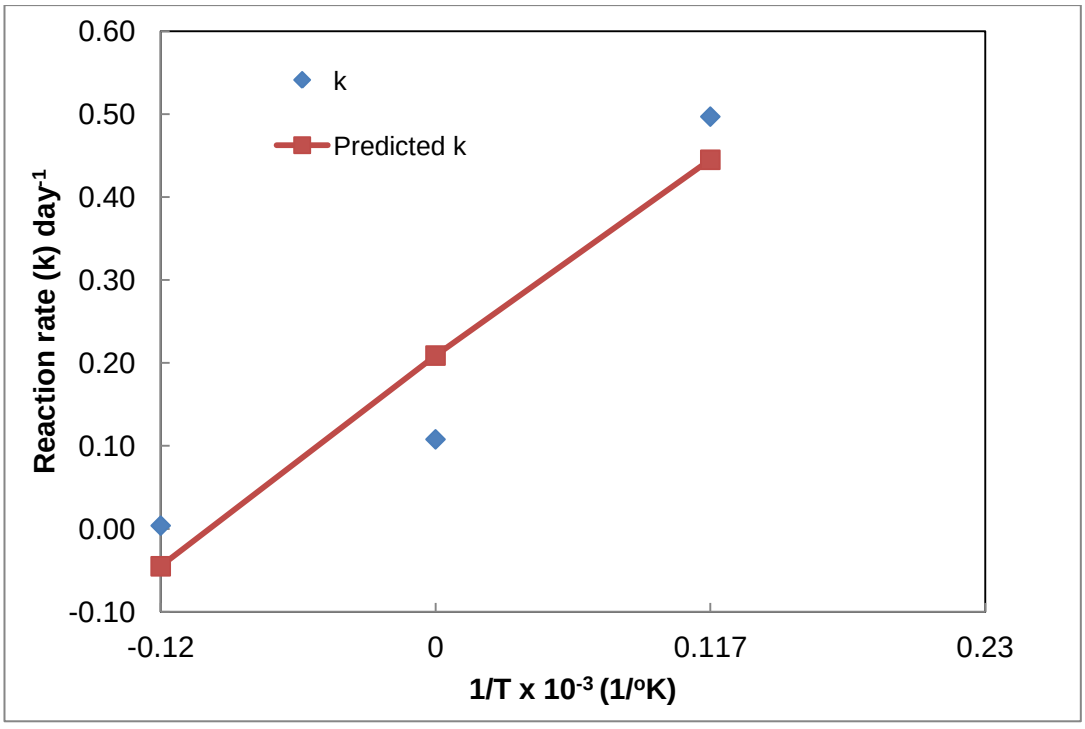

(b)

Figure 5.7 Arrhenius plot of reaction rate constant for pH changes during storage (a) *L. bulgaricus* and (b) *L. bulgaricus* and *L. plantarum* BGNPB

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Table 5.3 Parameters of Arrhenius equation for the pH of BGNPB during storage

Probiotic	Temperature (^{o}C)	E_a (kJ/mol)	k_{qref}	R^2
L. bulgaricus	5-25	12.8 ± 6.5	1.26 ± 1.08	0.80
L. bulgaricus & L. plantarum	5-25	16.9 ± 6.0	1.23 ± 1.07	0.89

BGNPB – Bambara groundnut probiotic beverage

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this study was to assess bambara groundnut milk (BGNM) fermented with lactic acid bacteria (LAB) as a probiotic beverage with a view to developing value-added product. This objective was achieved by undertaking the following: Optimised BGNM production followed by the production of consumer acceptable BGNM; optimised the survival of LAB in BGNM with a view to identifying the best strain for fermentation and developed BGN probiotic beverage (BGNPB) with viable cell count not less than 10^7 cfu per mL at the end of shelf life according to the CODEX Standards for fermented milk (Anon, 2003). The BGNPB was evaluated for LAB survival in simulated gastric juice and bile tolerance.

BGNM was produced from BGN flour by hydrating the flour at 25^{o}C for 2 h. The pH, hue and total solids of the milk were estimated to be 6.52, 54^o and 2.6, respectively. The possibility of producing BGNM from hydrated flour rather than through wet milling and dehulling is beneficial as (1) there is huge saving on energy; (2) the milk will contain all the goodness in the seeds especially the pigments on hulls which includes phenolics and flavonoids which may contribute to nutritional well-being. Quality of BGNM was affected by variety in terms of pH, colour and antioxidant activity. Milk from black, brown and red had less hue and high in yellowness, while black-eye and brown-eye had high hue but less in yellowness. Variety also affected consumer acceptability of BGNM. Taste, appearance, and colour were the important parameters considered by the panellist in their preference. In terms of variety, the BGNM from red (74.4%) and the brown (53.8%) were moderately undesirable in taste and moderately desirable both in appearance and colour. Preference for BGNM was high among all the age groups, demonstrating the potential of BGNM when commercialised.

Strain linked variability of acidification rate resulted in BGNPB with varying physical properties. BGNPB could be classified into two groups on sensory basis. The consumers moderately desired the beverage in taste and mouthfeel. Sucrose also affected consumer acceptability of BGNPB beverage. *L. bulgaricus* being responsible for synthesising of aromatic compounds, fermented BGNM had the

normal yoghurt flavour. Taste, appearance and colour were the important parameters considered by the panellist in their preference. Preference for BGNPB was high among the > 40 age groups, demonstrating the potential of BGNPB when more sucrose is added and commercialised.

The seeds of BGN are considered a complete food as they contain sufficient amounts of the essential nutrients. Their product the BGNPB showed to be nutritious, and could supply an average of 11.5% of the recommended daily allowance for proteins. The *in vitro* results suggested that the probiotics in the beverage can successfully transit the human stomach reaching the colon in relative effective numbers. Using changes in pH as the index of quality, kinetics of pH was modelled using Mitscherlich's law of diminishing returns model and effect of temperature on rate of reaction using the Arrhenius equation. Kinetics studies showed that pH of BGNPB was relatively stable under refrigeration (5^oC), resulting in a right censored shelf life of 28 days; 18 and 10 days, respectively for *L. bulgaricus* and *L. bulgaricus* & *L. plantarum* BGNPB under abused temperature (15^oC) and 2 days at accelerated storage temperature (25^oC) for both variants. To exert the beneficial effects on the host, a probiotic product is expected to have 10^7 cfu mL^{-1} at the end of shelf life. The BGNPB had 10^7 cfu mL^{-1} at the end of 28 days storage at 5^oC making it an effective probiotic beverage. Furthermore, shelf life of the BGNPB under refrigeration (5^oC) was high when compared to their dairy counterparts with a shelf life of 14 days. The following conclusions can therefore be made from this study:

1. BGNM can be produced from whole BGN flour by hydrating the flour for 2 h at 25^oC.
2. BGNM can support the growth of LAB with optimum growth at 35^oC for 24 h for *L. bulgaricus* and *L. plantarum*.
3. Consumer acceptable BGNPB could be produced with *L. bulgaricus* and in combination with *L. plantarum* (1:1).
4. That BGNPB could safely be stored up to 28 days at refrigeration temperature (5^oC) with a probiotic load of 10^7 cfu mL^{-1}.
5. BGNM is an alternative probiotic carrier and can be a solution to consumers demanding health foods that not only look nice but also taste well and have an appropriate shelf life. BGNPB is a combination of nutrition and

innovation, making it an ideal concept and development candidate for industrial scale producers.

6. BGNPB has the potential to improve food security in many rural areas as well as become a stable, low-cost protein source and profitable food crop for Africa's small-scale farmers as well a new source of food ingredient for the food industry.
7. South Africa- Provisional Patent (**PA154380/P)** for Bambara Probiotic Beverage from this work was filed on the 20 January 2012 (Appendix 8).
8. A manuscript written from this study has been accepted for publication in Critical Reviews in Food Science and Nutrition (Appendix 9).

APPENDICES

Appendix 1 ANOVA for pH – Optimisation of BGNM production

Source	Sum of squares	df	Mean square	F value	p-value
Block	0.18	3	0.059		
Model	0.017	4	$4.14x10^{-3}$	11.61	0.0001
Temperature	$2.853x10^{-3}$	1	$2.853x10^{-3}$	7.99	0.0067
Time	$5.32x10^{-3}$	1	$5.32x10^{-3}$	14.91	0.0003
*Temperature*Time	$2.4x10^{-3}$	1	$2.4x10^{-3}$	6.72	0.0123
$Time^2$	$3.501x10^{-3}$	1	$3.501x10^{-3}$	9.81	0.0029
Residual	0.019	52	$3.570x10^{-3}$		
Lack of fit	$1.499x10^{-3}$	1	$1.499x10^{-3}$	4.48	0.0392
Pure error	0.017	51	$3.346x10^{-4}$		
Correct Total	0.21	59			

Appendix 2 ANOVA for lightness – Optimisation of BGNM production

Source	Sum of squares	df	Mean square	F value	p-value
Block	134.60	3	44.87		
Model	87.84	3	29.28	12.57	0.0001
Temperature	0..050	1	0.050	0.022	0.8834
Time	83.62	1	83.62	35.90	0.0001
*Temperature*Time	2.43	1	2.43	1.04	0.3109
residual	170.03	73	2.33		
Lack of fit	120.38	2	24.83	14.64	0.0001
Pure error	120.38	71	1.70		
	392.47	79			

Appendix 3 ANOVA for hue – Optimisation of BGNM production

Source	Sum of squares	df	Mean square	F value	p-value
Block	93.03	3	31.01		
Model	278.44	3	92.81	4,29	0.0076
Temperature	23.64	1	23.64	1.09	0.2992
Time	94.81	1	94.81	4.38	0.0398
*Temperature*Time	172.98	1	172.89	8.00	0.0060
Residual	1578.67	73	21.63		
Lack of fit	0.70	2	0.35	0.016	0.9845
Pure error	1577.97	71	22.22		
Cor Total	1950.13	79			

Apendix 4 ANOVA for total solids – Optimisation of BGNM production

Source	Sum of squares	df	Mean square	F value	p-value
Block	5.39	3	1.80		
Model	2.42	2	1.21	7.28	0.0023
Temperature	1.28	1	1.28	7.71	0.0089
Time	0.74	11	0.74	4.45	0.0423
Residual	5.65	34	0.17		
Lack of fit	1.40	3	0.47	3.39	0.0302
Pure error	4.26	31	0.14		
Cor Total	13.46	39			

Appendix 5 ANOVA for the response of pH on BGNM

Source	Sum of squares	df	Mean square	F value	p-value
Model	106.03	14	7.57	71.60	0.0001
Probiotics	36.93	3	12.31	116.37	0.0001
Temperature	0.054	1	0.054	0.51	0.4767
Time	49.44	1	49.44	467.40	0.0001
*Probiotics*Temp	12.94	3	4.31	40.78	0.0001
*Probiotics*Time	5.34	3	1.78	16.81	0.0001
*Temperature*Time	2.723×10^{-4}	1	2.723×10^{-4}	2.574×10^{-3}	0.9596
$Temperature^2$	0.55	1	0.55	5.16	0.0242
$Time^2$	0.20	1	0.20	1.89	0.1705
Residual	21.26	201	0.11		
Lack of fit	16.66	21	0.79	31.07	0.0001
Pure error	4.60	180	0.026		
Cor Total	127.29	215			

Appendix 6 ANOVA for the response of lactic acid on BGNM

Source	Sum of squares	df	Mean square	F value	p-value
Model	11.83	14	0.85	22.21	0.0001
Probiotics	2.20	3	0.73	19.29	0.0001
Temperature	0.052	1	0.052	1.37	0.2472
Time	4.22	1	4.22	111.05	0.0001
*Probiotics*Temp	1.90	3	0.63	16.61	0.0001
*Probiotics*Time	3.20	3	1.07	28.07	0.0001
*Temperature*Time	3.2×10^{-3}	1	3.2×10^{-3}	0.084	0.7728
$Temperature^2$	0.054	1	0.054	1.41	0.2398
$Time^2$	0.20	1	0.20	5.17	0.0268
Residual	2.17	57	0.038		
Lack of fit	1.69	21	0.080	5.98	0.0001
Pure error	0.48	36	0.013		
Cor Total	14.00	71			

Appendix 7 ANOVA for the response on OD for the BGNM

Source	Sum of squares	df	Mean square	F value	p-value
Model	0.67	14	0.048	15.98	0.0001
Probiotics	0.25	3	0.083	27.73	0.0001
Temperature	9.718×10^{-4}	1	9.718×10^{-4}	0.33	0.5687
Time	0.23	1	0.23	78.33	0.0001
*Probiotics*Temp	0.058	3	0.019	6.44	0.0004
*Probiotics*Time	0.055	3	0.018	6.18	0.0006
*Temperature*Time	5.711×10^{-3}	1	5.711×10^{-3}	1.92	0.1683
$Temperature^2$	4.789×10^{-4}	1	4.789×10^{-4}	0.16	0.6890
$Time^2$	0.066	1	0.066	22.07	0.0001
Residual	0.39	131	2.976×10^{-3}		
Lack of fit	0.19	21	9.191×10^{-3}	5.13	0.0001
Pure error	0.2	110	1.790×10^{-3}		
Cor Total	1.06	145			

Appendix 8- South Africa- Provisional Patent Application

Appendix 9

Manuscript Accepted for Publication in Critical Reviews in Food Science & Nutrition