Sudan University of Science and Technology (SUST)
College of Graduate Studies

The Potential Formulation of Malted Barley (*Hordeum Vulgare* L.) Beverages Fermented with *Bifidobacterium longum* BB536 as a Functional Food

إمكانية تكوين خلطة من مشروب الشعير المنبت (*Hordeum vulgare* L.) والمخمر بالباكتيريا *Bifidobacterium longum* BB536 غذاءً وظيفياً

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الإستهلال

بسم الله الرحمن الرحيم

قال تعالى:

(فَلْيَنْظُرِ الْإِنْسَانُ إِلَى طَعَامِهِ *أَنَّا صَبَبْنَا الْمَاءَ صَبًّا *ثُمَّ شَقَقْنَا الْأَرْضَ شَقًّا * فَأَنْبَتْنَا فِيهَا حَبًّا* وَعِنَبًا وَقَضْبًا* وَزَيْتُونًا وَنَخْلًا* وَحَدَائِقَ غُلْبًا* وَفَاكِهَةً وَأَبًّا* مَتَاعًا لَكُمْ وَلِأَنْعَامِكُمْ*

صدق الله العظيم

سورة عبس الآيات (24-32)

Dedication

To My Father

To My Mother

To My Uncle

To My Wife

To My Children

To My Brothers

To My Sisters

To My Teachers

and to my all Friends

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Abstract

This study aimed to determine the nutritional composition and antioxidant profile of different malted barley varieties and formulation fermented beverages by *Bifidobacterium longum* BB536 as functional food via exploration using Albino rats.Three varieties of barley grains varieties from Yemen (Bukur and Balady) and Sudan (Local 46) were used. Malting barley grains were cleaned, soaked, germinated (30±2ºC for 120 hours), and dried (55 ºC for 12 hours). Beverages were prepared by blending 10% barley (w/v) for 5 mins at medium speed and filtered the slurry by double layer cheese cloths. On the other hand, 10% Re-constituted skim milk (w/v) was used as a control. All barley beverages including the control were sterilized (121°C for 15 mins), inoculated with 3% (w/w) active culture of *B. longum* BB536, and fermented by incubation at 37°C for 24h. Different analyses were carried out include: proximate composition (Moisture, Protein, Fat, Fiber, Ash and Carbohydrate), minerals (Na, Ca, P, K, N, Cu, Fe, Zn and Mg), sugar (total sugar, reducing and Non-reducing sugar), antioxidant profile (polyphenol, flavinol, tannin and phtate), safety (total arobic, yeast and molds, total coliform, *E.coli* and *Salmonella*), strain BB536 growth, physiochemical properties (pH, total soluble solids and acidity) and senosery evaluation (colour, taste, flavour, odor and overall acceptability). Further the effect of the fermented malted barley beverages on intestinal microflora (*Lactobacillius* and *Bifidobacterium*) and general health including (weight gain, feed intake and water consumption), haematology and blood biochemistry was explored on 30 (five different groups, n=6) male Albino rats after acclimatization 15 days under to the experimental conditions and oral feeding for a period of 30 days.The results ofthe three barley varieties (Bukur, Balady and Local 46) revealed significant differences ($p<0.05$) in chemical composition, minerals and antioxidant profile between non malted and non malted varieties. Safety analysis

of malting samples showed that the total count of bacteria and yeasts and moulds were within the standard of SSMO (Sudanese Standards and Metrology Organization). However, total coliform, *E.coli and Salmonella* were detected in malted samples. At fermentation, *B. longum* BB536 viable count was significant (P<0.05) increase and reached the highest level of 8.13, 8.21, 8.08 and 8.14 log CFU/ml, in control, Bukur, Balady and Local 46 beverages, respectively. All beverages contained more than 6 log BB536 CFU/ml exceed which the minimum number required in probiotics foods to exert health benefits. At 3 week refrigeration of fermented beverages there were significant (p<0.05) reductions in *B. longum* BB536 viable count, pH, TSS, and total sugars. Yeast and mould, *Staphylococcus*, *Salmonella* and *E. coli*, were not found in all fermented beverages.Thus, they are safe for human consumption. Overall acceptability of fermented barley beverages affirmed that panelists mostly preferred Re-consisted skim milk followed by Bukur fermented beverage and then Balady fermented beverage. *In vivo* (rat fed fermented beverages) positively affected health of rats. There were no blood haematology abnormalities and no signs of any moderate and mild deficiencies of nutrients revealed in rat blood. Moreover, serum electrolytes and enzymes of liver such as ALT, AST and ALP of fed rats groups were within the standard normal ranges. Blood of rats groups fed fermented barley products recorded the lowest cholesterol, triglyceride and glucose levels in all group compared to control. In addition, *Bifidobacteria* and *Lactobacillus* of colon increased thus caused decreased of pathogens (*Staphylococcus*, *Salmonella*, and coliform). Therefore, the developed malted barley beverage is a probiotic products carried *B. longum* BB536 at high levels and showed positive signs on rats health thus, it consider as a functional food for human.

ملخص البحث

هدفت هذة الدراسة الي تقدير القيمة الغذائية ومضادات الأكسدة في أصناف مختلفة من الشعير المنبت، وتصنيع شراب شعير مخمر وظيفي بواسطة *Bifidobacterium longum* BB536، واستكشاف أثرة على فئران التجارب. تم جمع ثلاثة أصناف من حبوب الشعير؛ صنفين من اليمن (بكور وبلدي)،بجانب صنف سوداني (محلي 46). تم تنظيف حبوب الشعير للإنبات حيث تم نقعها في الماء لمدة 24 ساعة وتركت للانبات على درجة حرارة (30°م لمدة 120 ساعة) ثم جففت على درجة حرارة (55° م لمدة 12 ساعة). حُضر مشروب الشعير المنبت المخمر عن طريق خلط 10% من مسحوق الشعير مع 90% ماء مقطر(وزن/حجم) لمدة 5 دقائق على سرعة متوسطة، بعد ذلك رشح الخليط بواسطة قطعة قماش مدبلة المستخدمة في صناعة الجبن. من جهة أخرى تم تحضير العينة الضابطة بواسطة خلط 10% من الحليب المنزوع الدسم مع 90% ماء مقطر (وزن/حجم). عُقمت جميع المشروبات المحضرة من أصناف الشعير الثلاثة بجانب مشروب العينة الضابطة على درجة حرارة121°م تحت ضغط 15 رطل على بوصة المربعة لمدة 15 دقيقة. بعد ذلك لقحت جميع المشروبات بواسطة 3%BB536 النشطة (حجم /حجم) وتركت بالحضان على درجة حرارة 37°م لمدة 24 ساعة لغرض تخميرها.

أجريت العديد من التحاليل والتي شملت التحليل التقريبي(الرطوبة، البروتين، الدهن، الرماد، الالياف والكربوهيدرات)، مضادات الأكسدة (البولي فينولينات، الفلافونيدات، التانينات والفيتات)، المعادن (الصوديوم، الكالسيوم، البوتاسيوم، الحديد، النحاس، الزنك، المنجنيز، الماغنسيوم)، والسكريات (السكريات الكلية، السكريات المختزلة وغير المختزلة)، والسلامة الميكروبية (العد الكلي للبكتيريا، الفطريات والخمائر، الايشيريشيا كولاي، مجموعة الكوليفورم والسالمونيلا). كذلك أجريت أختبارات العد الكلي للبفيدوباكتريوم والخواص الفيزوكيميائية (الاس الهايدروجيني، الحموضة، المواد الصلبة الذائبة) لمشروبات الشعير المخمرة المختلفة، وأخيرا إجراء التقييم الحسي (اللون، الطعم، النكهة، الرائحة، القبول العام). تم التحقق من تأثير مشروب الشعير المخمر على بكتيريا الامعاء (الميكروفلورا) والصحة العامة (الوزن المتحصل علية، كمية الغذاء المتناول وكمية الماء المستهلك) بجانب دراسة أختبارات أمراض الدم والإختبارات الكيموحيوية للدم، والتي أجريت على ثلاثين فأرا من ذكور الألبينو بعد فترة تكييف المحددة ب 15 يوم. بعد ذلك تم تغذية الفئران بمشروب الشعير المخمر لمدة 30 يوماً.

أظهرت النتائج المتحصل عليها لإصناف الشعير الثلاثة (البكور، بلدي، محلي46) فروق معنوية من حيث التحاليل الكيميائية التقريبية، المعادن، السكريات و مضادات الاكسدة بين وبعد الإنبات لكل صنف على حده. العد الكلي والفطريات والخمائر في الأصناف المنبتة وغير المنبتة كانت ضمن الحدود القياسية،

كذلك تم الكشف على العد الكلي الكوليفورم الايشيريشيا والسالمونيلا. كانت هناك زيادة معنوية ($p<0.05$) في عدد النمو لسلالة BB536 في كل أنواع المشروبات المصممة مقارنة ببداية التخمر، وكانت العد الكلي (8.13، 8.21، 8.08 و8.14 وحدة مكونة مستعمرة/مل) ل العينة الضابطة، البكور، بلدي و محلي46 على التوالي. الحد الاعلى للبكتيريا الصديقة BB536 في الأربعة الأنواع من المشروبات كان أكثر من 6 وحدة مكونة مستعمرة/مل والذي يوفر الحد الادني للعدد المطلوب وجوده في أغذية البكتيريا الصديقة لإعطاء فوائد صحية للإنسان. أثناء التخزين المبرد لمختلف أصناف المشروبات المصممة كان هناك أنخفاض معنوي ($p<0.05$) في أعدادBB536 وpH والجوامد الصلبة الكلية، والسكريات الإجمالية في التخزين لمدة ثلاث أسابيع لجميع المشروبات المخمرة. كما أوضحت النتائج خلو المشروبات المخمرة من الخمائر والفطريات، الاستافيلوكوكس، السالمونيلا، والايشيريشيا كولاي وبالتالي فهي أمنه للاستهلاك الأدمي. وقد أوضح القبول العام لمختلف أنواع مشروبات الشعير المخمرة بسلالة BB536 أن معظم المشاركين فضلوا العينة الضابطة يليها مشروب الشعير من صنف بكور ثم بلدي وأخيرا محلي 46. أما من حيث تغذية فئران التجارب على مشروبات الشعير المخمرة المختلفة فإن نتائج التحاليل أشارت إلى نتائج إيجابية في صحة الفئران. ومع ذلك، لم يكن هناك خلل في أمراض الدم ولاتوجد علامات على أي نقص معتدل أو خفيف في الموادالغذائية. كما يتضح من النتائج الكيميائية حيوية في الدم كانت أنزيمات الكبد مثل ALT وAST وALP من مجموعات الفئران المغذية ضمن النطاق الطبيعي القياسي للفئران.
كما أظهرت نتائج التحاليل الكيموحيوية للدم في الفئران المتغذية على منتج الشعير المخمر إنخفاضا معنويا ($p<0.05$) في مستوى الكوليسترول والدهون الثلاثية (الجليسريدات الثلاثية) ومستوى الجلوكوز في الدم.
تغذية فئران التجارب بمشروبات الشعير المخمرة قد حفزعلى على زيادة معنوية ($p<0.05$) في نمو *Bifidobacterium longum* BB536 مقارنة بالمجموعة الضابطه في القولون وكذلك زيادة بكتيريا *Lactobacillus* في كل مجموعات الفئران مقارنة مع الضابطه. بالإضافة إلى ذلك كان هناك إنخفاض ملحوظ في البكتيريا الممرضة مثل السالمونيلا والمكورات العنقودية. وعلية تم الاستنتاج أن مشروب الشعير المخمر قد حفز نمو البكتيريا الصديقة *Bifidobacterium longum* BB536 وأيضاً أظهرآثار إيجابية في الفئران مما يؤدي الي أهميتة كغذاء وظيفي.

CHAPTER ONE

INTRODUCTION

Cereal grains are the most important source of the world's food and have a significant role in human diet through out the world. Barley (*Hordeum vulgare*), like all other true cereals, is a member of the grass family including wheat, maize, rice, rye, millet, oats, sorghum and triticale. They are the nine most important cereals grown in the world today. Globally, barley is the fourth most produced cereal, after maize, rice and wheat (FAO STAT, 2007). In addition among cereals, barley is an excellent source of soluble and insoluble dietary fiber and other bioactive constituents, such as vitamin E, B-complex vitamins, enzymes, minerals, and phenolic compounds (Gamlath *et al.,* 2008). It has one of the highest levels (up to 6%) of β-glucan, a water-soluble polysaccharide nutritionally classified as soluble dietary fiber (Izydorczyk and Dexter, 2008). On the other hand, malting is the process of controlling germination of cereal grain followed by drying of the seed developed. It can be applied to any type of cereal grain, but barley has been the most commonly used cereal for malting process. Nowadays, consumers health awareness positively directed towards the intake of foods low in fats, cholesterol and salt worldwide. In Sudan the fermentation process is considered the most important methods of food preparation and preservation (Dirar, 1993). That is because fermented foods have an enhanced nutritional value, digestibility, better flavor, improved appearance, reduced cooking time, and good texture (Chavan*et al.,* 1988). However, the high viscosity of cereal based beverage might hinder the growth of bacteria during fermentation process. The addition of amylase enzyme or malted cereal grains including malted rice was found to be successful in reducing the viscosity or liquefy the beverage (Flowing characteristics) hance facilitate microbial biomass. Normally,

conventional starter cultures are used for cereal food fermentation. Nevertheless, utilization of friendly beneficial bacteria in food process such as probiotics in cereal food fermentation could add health benefical value is widely encouraged (Kabeir *et al.,* 2005).

Probiotics are live microorganisms that when ingested in adequate amount confer a health benefit to host (FAO/WHO, 2001). Non-dairy probiotic products have shown a big interest among vegetarians and lactose intolerance customers. According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the U.S. National Institutes of Health, about 75% of the world population was lactose intolerant. The development of new non-dairy probiotic food products was very much challenging, as it has to meet the consumer's expectancy for healthy benefits (Stanton *et al.,* 2003).

Among probiotics, *Bifidobacterium longum BB536* is successfully approved strain and has been found mainly in human feces and it may be considered as the most common species of *Bifidobacterium* being found both in infant and adult. Potential health benefits from consumption of *B. longum* include: antagonistic action toward intestinal pathogens, improved lactose utilization, anticarcinogenic action and control of serum cholesterol levels. Scientific studies showed the benefits offered by *Bifidobacterium longum* BB536 (Nambak *et al*., 2003). These apprenced scienfic evidences increased the health's promoting *Bifidobacterium* strain into human food.

Bifidobacterium species are gram positive, non-spore forming, non-motile rods with high GC content; they are anaerobic to aerotolerant and are generally catalaze negative. They are able to ferment glucose to lactic and acetic acids through the F6PPK-pathway (fructose-6-phosphate phosphoketolase). Moreover,

many studies have been reported on incorporation of *Bifidobacterium* in different cereal but not many studies were conducted on barley.

General objective

To develop functional *Bifidobacterium longum* BB536 fermented malted barley beverages and assessment of their beneficial effects on in Vivo exploration using rats.

Specific objectives

1- To the determine the effect of malting on chemical composition, mineral and total sugars of malted barley.

2- To evaluate safety of malted barley.

3- To establish the effect of malting on phenolic compound and flavonoid of barley varieties.

4- To calculate the growth of probiotic *Biofidobacterium longum* BB536 during fermentation of different malted barley varieties and assess strain survival during refrigeration storage of the fermented beverages.

5- To determine chemical composition, physicochemical changes (pH, TSS, Titerable Acidity and sugars) of the different strain BB536 fermented barley varieties.

6- To explore the effect of feeding fermented barley beverage on general health (Weight gain, food intake and water consumption), blood haematology and biochemical of fed albino rats.

7- To examine probiotic effect of feeding strain BB536 fermented barley varieties beverages on intestinal microbial comunities of fed rats.

CHAPTER TWO
LITERATURE REVIEW

2.1. Cereals

Cereals are defined as grains or edible seeds of the grass family, Gramineae (Bender and Bender, 1999). They are grown for their highly nutritious edible seeds, which are often referred to as grains. Some cereals have been staple foods both directly for human consumption and indirectly via livestock feed since the beginning of civilisation (BNF, 1994). They have a long history of use by humans as important sources of nutrients in both developed and developing countries for their high energy, carbohydrate, protein, fibre, vitamins (E and B), mimerals such as magnesium and zinc (FAO, 2002). Generally, cereals are cheap to produce, easily to store and transport, and do not deteriorate readily if kept dry.

2.2. Types of cereal grains

The three most important food crops in the world are rice, wheat, and maize (corn). The three cereal grains directly contribute more than half of all calories consumed by human beings. In addition, other minor grains like sorghum and millet are particularly major contributors of overall calorie intake in certain regions of the world, particularly semi-arid parts of Africa and India. For example, sorghum and millet contribute up to 85% of daily caloric intake in Burkina Faso and Niger (FAO, 2011). A large part of cereal grain production (particularly corn, barley, sorghum, and oats) also go into livestock feed, thus indirectly contributing to human nutrition.

2.3. Barley

Barley belongs to the family Poaceae and the genus *Hordeum*. The most common form of barley is *Hordeum vulgare*. It can be classified as spring or winter types, two-row or six-row (in two-row barley only the central spikelet is fertile, while

in six-row barley has fertile lateral spikelets also), hulled or hull-less (which relates to the presence or absence of an outer husk attached to the grain) and malting or feed (which relates to its end-use). The composition of barley grain can be classified as normal, waxy or high amylose starch types, high lysine, high β-glucan or proanthocyanidin-free. Barleys of different classes often differ widely in both their physical and compositional characteristics, and as a result they have different processing properties and end-uses. Barley has been reported to contain a number of essential amino acids, including threonine, valine, lysine and arginine. Essential amino acids are amino acids that cannot be made by the human body, or cannot be produced fast enough to meet the body's demand for them. These amino acids therefore, must be supplied by the diet as they play important roles in metabolic pathways. For example, arginine is involved in the synthesis of urea in the liver, while lysine is involved in the production of creatinine, which is a substance that transports fatty acids within cells (Sullivan, 2010).

2.3.1. Barley taxonomy

Barley (*Hordeum vulgare* L.*)* is a member of the *triticeae* tribe of grass family *Poaceae* (*Gramineae)*. All the *Poaceae* members have likely evolved from a common ancestor (Devos, 2005), thus the Poaceae family can be considered monophyletic. The *Triticeae* tribe consists of 350 to 500 species. Among which several important cereal and forage crops such as wheat (*Triticum spp.*), rye (*Secale cereale* L.) and crested wheat grass (*Agropyron cristatum*). However, the taxonomic delimitation of the tribe has not been fully resolved.*Hordeum* comprises of a group of well-defined and easily recognized plants made up of 32 species and 45 taxa with the basic chromosome number of seven. Most of the *Hordeum* members are diploid (2n = 2x = 14), tetraploid (2n = 4x = 28) and

hexaploid (2n = 6x = 42) (Bothmer *et al.,* 2003). Two species, *H. bulbosum* L. and *H. brevisubulatum* L. are autoploids and a few anploid *Hordeum species* have evolved due to elimination or duplication of chromosomes (Linde-Laursen *et al.,* 1986). The two-row shattering (*H. spontaneum* K.) and non-shattering (*H. distichum* L) barley and the six-row shattering (*Hordeum vulgare* L.) and non-shattering (*H. agriocrithon* E. Åberg) barley were previously considered as four separate species. Today, they are all known as *Hordeum vulgare* L. After the discoveries that the differences in shattering and spike types are due to two complementary brittle rachis (*Btr*) genes (Zohary and Hopf, 2000). The shattering character of *H. spontaneum* is caused by brittle rachis which enables seed dispersal in the wild. Another form of shattering occurs when breaking of rachis leads to complete loss of spike. A mutation in one or both of the tightly linked *Btr1* and *Btr2* on chromosome 3H resulted in development of shattering-resistant barley (Azhaguvel *et al.,* 2006). The cultivated form of barley (*Hordeum vulgare* L.) also includes a group of barley lines derived from crosses between two-row and six-row barley that were previously denoted as *H. intermedium* (Jui *et al.,* 1997). Cultivated barley is quite similar to its wild progenitor *H.* s*pontaneum,* with the exception of having broader leaves, tougher ear rachis, shorter and thicker spike, larger grains and shorter stems and awns.

Hordeum genus is widespread in temperate areas and occurs in several biotopes worldwide. Most native *Hordeum* species have their diversity centers in southern South America, western North America, the Mediterranean, and Central Asia (Bothmer *et al.,* 2003). The global barley inventory held by centres such as the International Center for Agricultural Research in the Dry Areas, Aleppo, Syria includes nearly 12,500 accessions of *H. vulgare* subsp. *spontaneum* originating

from 25 countries concentrated to the western part of the Fertile Crescent (Jilal *et al.,* 2008).

2.4. Chemical composition of barley

2.4.1. Carbohydrates

Carbohydrates are organic molecules with the general formula $C_x(H_2O)_y$. Simple sugars like glucose, fructose and galactose make up the building blocks of carbohydrates, which can be classified as monosaccharides, disaccharides, oligosaccharides or polysaccharides (Chibbar *et al.,* 2004). Carbohydrates are the main energy source for humans, and can from a nutritional aspect be divided into available carbohydrates (readily digested in the small intestine) and unavailable carbohydrates (not digested in the small intestine but fermented in the large intestine). Glucose and fructose are examples of available carbohydrates, whereas many oligosaccharides, resistant starches, pectins, β-glucan, cellulosic and / non-cellulosic polysaccharides and hemicelluloses are regarded as unavailable (Chibbar *et al.,* 2004).

2.4.1.1. Monosaccharides

The mature endosperm of normal barley contains 2-3% monosaccharides, mostly in the form of glucose and fructose. Higher concentrations of simple sugars are found in hulless (2-4%), high lysine (2-6%) and high sugar (7-13%) barley grain (Holtekjølen *et al.,* 2008). Other monosaccharides such as fucose, arabinose, xylose, ribose, deoxyribose galactose and mannose are produced in grain to form oligo-and polysaccharides, glycosides, glyco-lipids or glyco-proteins (Holtekjolen *et al.,* 2008).

2.4.1.2. Disaccharides

The two most important disaccharides in barley grain are sucrose and maltose. The concentration of sucrose ranges from 0.74-0.84%, with close to 80% present

in the embryo (MacGregor and Fincher, 1993). Sucrose serves as an important precursor for starch biosynthesis, and can be accumulated to relatively high levels (7%) in waxy genotypes defective in amylose biosynthesis (Batra *et al.,* 1982). Maltose accumulates to concentrations of 0.1-0.2% in barley endosperm as a result of starch amylolytic activities (Sopanen and Lauriere, 1989). Due to increased starch-bound α-amylase activity in waxy genotypes, the maltose concentration can reach 0.4% (Nielsen *et al.,* 2009).

2.4.1.3. Oligosaccharides

Oligosaccharides are polymers of 3-20 glucose units (Chibbar *et al.,* 2004). Raffinose, myo-inositol, fructosans and bifurcose are some of the oligosaccharides present in barley grains. The raffinose concentration is 0.3 to 0.8%, and 80% of it occurs in quiescent embryos (Andersen *et al.,* 2005), where it has a role in seed desiccation and constitutes a carbon source at the early stages of seed germination (Sreenivasulu *et al.,* 2008). Barley grains also contain fructo-oligosaccharides (fructans) which contain up to ten fructosyl residues (Janthakahalli, 2004). Fructans enhance drought tolerance in barley (Janthakahalli, 2004) and higher oligosaccharides concentrations in barley grains enhance seed survival under adverse weather conditions (Bønsager *et al.,* 2010).

2.4.1.4. Polysaccharides

Polysaccharides are glucose polymers synthesized by plants as storage carbohydrates (starch and β-glucan) or as structural carbohydrates (cellulose, chitin and arabinoxylans) as stated by Chibbar *et al.* (2004).

2.4.1.5. Starch

Starch is the most predominant carbohydrate in barley (Asare *et al.,* 2011) and is generally regarded as the most important carbohydrate in the world (Anker-Nilssen *et al.,* 2006). Photosynthesis in green leaves produces transitory starch

which is transported to the endosperm where it serves as the plants main energy reserve. In the endosperm starch is laid down in the endosperm tissue and stored as water insoluble granules. Two types and sizes of these water insoluble starch granules are found in the barley endosperm. The first type is a large lens-shaped granule which is initially formed by the amyloplasts that are found in barley. These lens shaped granules form outgrowths which separate from the amyloplast and form the second type of granule: a small spherical granule (Delcour and Hoseney, 2010).

2.4.1.5.1. Composition of starch

Most common starches are predominantly composed of polymeric carbohydrate material. This polymeric material is built up of monomeric -D-glucopyranosyl units which are linked to their neighboring glucose via glycosidic bonds. These glycosidic bonds have the orientation and are linked to either position 4 or position 6 on the glucose molecule. In general -1,4 bonds are thought to result in linear structures while -1,6 bonds result in a branched structure (Delcour and Hoseney, 2010). The two main types of polymers found in starch are amylose and amylopectin (Zobel, 1988). Amylose constitutes 20-25% of starch and is composed of a linear chain of -D-glucose units linked together by -1,4 bonds (Nybraaten, 2004). These -1,4 bonds give rise to a gradual left handed twist in the amylose chain, resulting in a spiral or -helix formation (Zobel, 1988). This -helix formation allows amylose to form complexes with a variety of chemical compounds such as iodine, fatty acids and alcohols. This is due to the fact that these compounds can position themselves inside the spiral formation and thereby interact with the amylose molecule (Nybraaten, 2004). In barley the amylose content can range from 3 to 46 % of the total starch content, with a normal level of approximately 20–30 % (Stevnebo *et al.,* 2006).The second main type of

polymer which is found in starch is amylopectin (Zobel, 1988). Amylopectin is one of the largest natural polymers (Delcour and Hoseney, 2010) and makes up 75-80% of starch. Similar to amylose, amylopectin is composed of a linear chain of –Dglucose units linked together by -1,4 glycosidic bonds (Nybraten, 2004). These linear chains are joined via -1, 6 glycosidic bonds and it is these linkages which give amylopectin its characteristic branched structure. Branching makes the amylopectin molecule very compact. Three types of chains are found in the amylopectin molecule; A-chains, B-chains and C-chains. The A-chains are composed of -1, 4-linked glucose units and are therefore not branched. The B-chains are made up of both - 1,4 and -1,6 glucose linked units and therefore carry branches. The C-chains is also branched and is composed of both - 1,4 and -1,6 glucose linked units. The C-chain is the only chain containing a reducing end in the amylopectin molecule (Delcour and Hoseney, 2010).

2.4.1.6. Non starch polysaccharides (NSP)

Non-starch polysaccharides such as β-glucan, arabinoxylans and cellulose are abundant in the cell walls of hull, endosperm tissue and aleurone layer of barley grains (Holtekjølen *et al.,* 2008). They are all classified as total dietary fiber (TDF). The β-glucan concentration for most barley genotypes ranges from 3-5%, however some hulless, waxy and high amylose genotypes have concentrations in 8-10% range (Izydorczyk *et al.,* 2000). Arabinoxylan concentration in barley kernel ranges from 3-5%. Cellulose is the primary component of barley hulls and the cellulose concentration in hulled and hulless barleys range from 4.1 to 4.8% and 2 to 2.9%, respectively (Holtekjølen *et al.,* 2008). Depending on end-use of barley grain, non-starch polysaccharides can have positive or negative effects. For feed and malting barley, relatively low beta-glucan concentration is required due to its adverse effect on feed energy value and malt quality. For human health,

high TDF is desired because of its beneficial effects on intestinal health and prevention of diabetes and cardiovascular diseases (Chibbar *et al.,* 2010). Balancing the concentrations of starch and non-starch components in the barley kernel will increase its utilization in feed, malt, food and other industrial purposes.

2.4.2. Protein

Barley kernel protein concentration is an important factor for malting, food and feed quality as proteins provide energy, nitrogen and catalyze many metabolic activities during seed germination. Some minerals such as calcium, iron, phosphorus and copper are attached to barley proteins and increase their availability during utilization.

The major storage proteins in barley endosperm are hordeins, which contain 35 to 50% of total grain nitrogen depending on grain protein content (Kirkman *et al.,* 1982). Hordeins are categorized as low-molecular-weight (LMW) hordeins (16.5 to 22 kDa) which include avenin-like proteins (A-proteins) and high moleculararg weight (HMW) hordeins (35 to 100 kDa) which include γ, B, C and D hordeins (Gubatz and Shewry, 2011). Grain with lower protein concentration will produce suboptimal enzyme levels during malting followed by poor yeast growth during brewing. When the protein concentration exceeds 12%, the yield of soluble substance decreases and malt quality is lowered. A long steeping time, erratic germination and haze in beer are other negative factors associated with high protein content in malting grain (Swanston and Molina-Cano, 2001).

2.4.3. Lipids

The lipids which are associated with cereal starches are generally polar and prevalent inside the starch granules. They consist mainly of lysophospholipids and unsaturated fatty acids; with linoleic acid (18:2) and palmitic acid (16:0)

being the two most abundant (Delcour and Hoseney, 2010). Some lipids are also found on the surface of the starch granule where they often act as a barrier to the digestion of starch. This is due to the fact that they diminish the contact between the digestive amylases and the substrate (Svihus *et al.,* 2005).

2.4.4. Vitamins

The vitamins found in the barley grain are often concentrated in the aleurone layer. Given the low level of lipids which are present in the barley grain, barley is a poor source of the fat soluble vitamins A, D and K. However, barley is an important source of the water soluble B- vitamins such as niacin, riboflavin and thiamin (Delcour and Hoseney, 2010).

2.4.5. Minerals

The mineral composition of the barley grain includes phosphorus, potassium, calcium, magnesium, iron, copper and manganesium (Delcour and Hoseney, 2010). The aleurone layer is the major storage site for the minerals phosphorus, magnesium, potassium, and calcium with over 70% of these minerals accumulating here (Becraft, 2007). The mineral content of barley grains varies from 2.0 to 3.0%, depending on genotype. Within the seed, ash is primarily located in the embryo, aleurone and pericarp tissues (Marconi *et al.,* 2000). As hull is rich in minerals (60 to70%), the ash content in hulled barley is higher than in hulless barley. Despite the low mineral content, hulless barley is preferred over hulled barley for feed of monogastric animals. Minerals which affect the nutritional value for the kernel are divided into macro and micro elements based on concentration in foods. The macro elements include calcium, phosphorus, potassium, magnesium and sodium. The rest are chloride, sulphur and silicon. Copper, iron, manganese, zinc, selenium and cobalt are the nutritionally important micro-elements in the barley kernel. Among the macro elements

phosphorus and potassium are the most abundant and in terms of nutritional qualities and availability. Phosphorus in barley kernel appears as phytic acid. Monogastric animals lack the enzyme phytase for phytic acid utilization. Higher concentration of phytic acid chelates other monovalent minerals such as calcium, copper and zinc making them unavailable (Delcour and Hoseney, 2010).

2.4.6. Enzymes

To release the energy which is stored as starch in the endosperm, the barley grain contains a number of enzymes. The starch-hydrolyzing enzyme α-amylase functions by hydrolyzing the α-1,4 linkages of the starch chain internally. This hydrolysis is more or less random and results in the production of α-limit dextrins, maltose and maltotriose. β-amylase produces the disaccharide maltose and is often referred to as the saccharifying or sugar producing enzyme. The combined activities of α-amylase and β-amylase degrade starch more rapidly and frequently than either of the enzymes working alone. However neither of these enzymes are capable of breaking the α-1,6 linkages present in amylopectin and therefore do not completely degrade starch. In general about 85% of starch is converted to sugar by a mixture of α- amylase and β-amylase. In addition to hydrolyzing the α-1,4 linkages at the non-reducing ends of the starch molecules, the exoamylase glucoamylase can break down the α-1,6 bonds in the amylopectin molecule. In theory glucoamylase can completely convert starch to β-D-glucose (Delcour and Hoseney, 2010).

2.5. Structure of barley grain

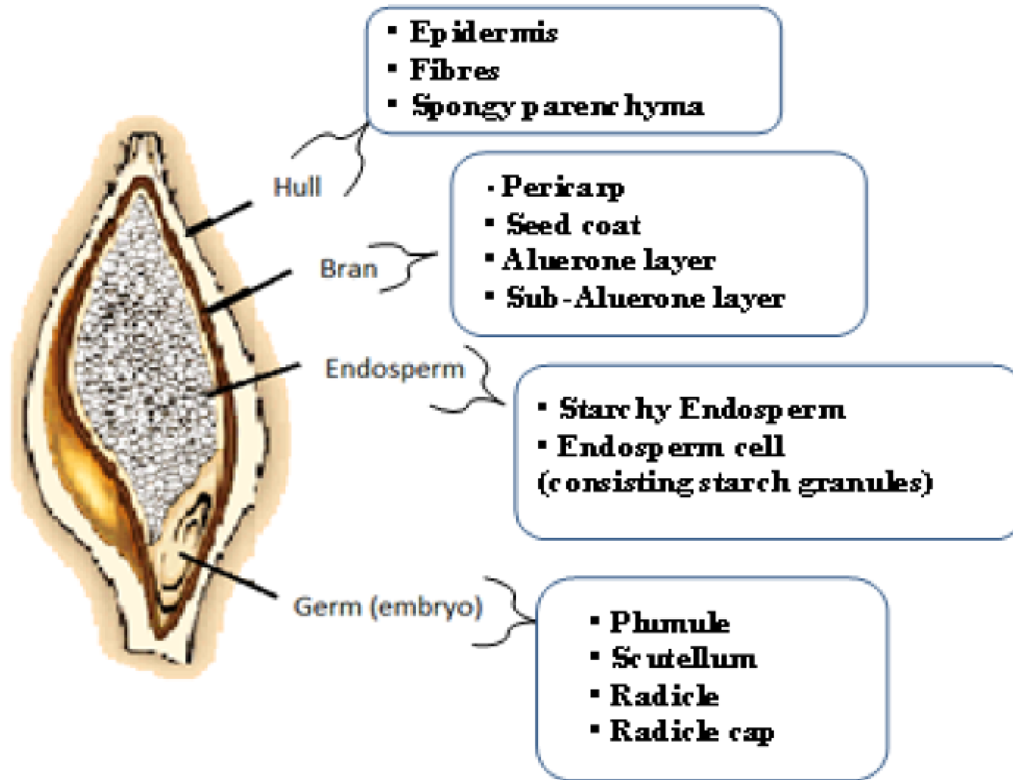

Figure 1.Stracture of barley grain

2.6. Bioactive compounds

Barley contains a high level of bioactive compounds including phenolic compounds such as benzoic and cinnamic acid derivatives, pro-anthocyandines, quinones, flavonols, chalcones, flavones, flavanones and amino phenolic compounds. The majority of these compounds are present in both free and bound forms but it is primarily the bound form which predominates in barley. The free phenolic compounds which have been identified in barley include proanthocyandines and flavonoids while the bound phenolic compounds consist mainly of ferulic acid and its derivatives (Bonoli *et al.*, 2004).

2.7. Malting process

Malting is a controlled germination process consisting of steeping or hydration of grains, a germination phase in moist conditions and finally the termination of the grain's physiological activities by heating during a phase called kilning. Fundamentally, the aim of malting is to unmask starch granules from the surrounding cell walls and protein matrix so that fermentable sugars can be optimally released from starch during the brewing process (Swanston *et al.,* 2014). Malt or malted barley is made from malting barley. To be classified as malting barley, the harvest from a malt barley variety has to meet strict specifications to ensure its quality. Malt extract is used around the world in many foods from cookies and cakes to beverages and baby foods to enhance flavour, colour, fermentation and aroma, as well as improve texture, shelf life and enrich nutritional content (Swanston *et al.,* 2014).

2.7.1. Steeping

The main objective of the steeping process is to rapidly induce barley germination without losing viability. This is accomplished by the immersion of grains in water which allows an increase in moisture content from 12 % to 42 – 48 %. Germination begins as the moisture content reaches 35%, but is increased to ensure uniform distribution of moisture and diffusion of enzymes throughout the endosperm. Temperature is maintained between 14 -18°C and the process usually takes 40–48h to complete. As steeping proceeds, water uptake slows down and dissolved oxygen is rapidly depleted in the steep water. This is due to increased metabolic activity of the grain and the microbial population on the surface tissue. To maintain germination vigour, the immersion phase (wet stand) is aerated and alternated with an air rest. Normally two to three alternating cycles are conducted throughout steeping (Booysen, 2001; Vaughan *et al.,* 2005). This technique

replenishes dissolved oxygen and allows the removal of accumulated carbon dioxide and ethanol that can cause water damage (Ullrich, 2011). Visual appearance of grains at the end of steeping is an important quality parameter. The grains are in the correct physiological state when the root sheath, also known as the chit, is presen (Booysen *et al.,* 2001; Ullrich, 2011).

2.7.2. Germination

During this phase the malts exploits the natural germination process, where by enzymes degrade the endosperm cellular structure. Germination is allowed to proceed only as far as necessary to ensure maximum fermentable products required by yeast during brewing. This is accomplished by carefully monitoring temperature, oxygen and carbon dioxide levels throughout the process (Ullrich, 2011). When germination is initiated, the grain undergoes extensive physiological and biochemical changes. The embryo secretes a plant hormone gibberellin, which triggers the scutellum and aleurone layer to produce hydrolytic enzymes (Palmer, 2006). These enzymes are deposited into the starchy endosperm where it attacks starch, cell wall polysaccharides and proteins (Jamar *et al.,* 2011). The biochemical degradation and physical weakening of the endosperm is generally referred to as modification. The hydrolyzed products diffuse back to the embryo to sustain its growth, although it is largely prevented during malting to avoid the depletion of nutrients that are essential for yeast fermentation. Grains are allowed togerminate for 4 - 6 days (Bamforth, 2000). Temperature is critical during this phase and is kept low (14 –18°C) to retard germination and ensure high nutrient levels for maximum yeast fermentation. At elevated temperatures germination is rapid and enzymes are produced at a premature stage. This in turn causes a greater loss in endosperm components due to sugar consumption by the embryo, and thus a reduced malt yield. As

germination proceeds, the embryo with draws moisture from the endosperm to sustain its growth. Approximately 0.5 % moisture is lost per day and humidified conditions are employed to prevent grains from drying out. Moisture content may also be retained by spraying grains with water Gibberellic acid (GA3) is applied by some maltsters as part of the water addition step. Germinating barley is referred to by the maltster as green malt and is ready to be kilned when acrospires reach 75% of the kernel length. It is crucial to prevent further acrospires elongation as this is a main factor that contributes to malt losses and are referred to as over modification (Ullrich, 2011).

2.7.3. Kilning

The malting process is finally terminated by kilning, during which the moisture level of green malt is reduced from 45 to 4 % (Wolf-Hall, 2007). The main objective of this phase is to arrest botanical growth and to preserve the majority hydrolytic enzymes required for further degradation of carbohydrates during mashing. Kilning also ensures microbial stability of malt and contributes to an assortment of colour and flavour compounds, which is mainly due to chemical changes during Maillard reactions (Noots *et al.,* 1998).The kilning process takes approximately 21 h during which temperature is gradually increased in a stepwise manner from about 50°C to 85°C with a reduction in airflow (Laitila *et al.,* 2007). After kilning is completed, rootlets are removed and the malt product is stored in silos (Booysen, 2001).

2.8. Biochemical changes during malting

Biochemically, malting is considered controlled processes of endosperm mobilization.These processes are initiated with steeping, reach maximal activity during germination and areterminated by the high temperatures and reduction in moisture content during kilning. Many of these processes are reinitiated when

malt grist is rehydrated during mashing. Modification is used to define the overall physical and biochemical changes that occur in the barleyendosperm during malting. Well-modified malt is friable and consequently easily crushed, whereas the opposite is true for poorly modified malt. This physical change is caused by the degradation ofcell walls and proteins within the endosperm (Jamar *et al.,* 2011).The extent of protein and cell wall degradation during malting is crucial to the maltster, as it determines the accessibility of starch to amylases, and consequently the extract yield during brewing. Incomplete modification leads to poor extract availability, while over-modification results in reduced malt extract yield as glucose liberated by starch degradation is consumed by embryo respiration (Ullrich, 2011). Germination is initiated as water enters the embryo during steeping. Gibberellic acid (GA3) is synthesized in the embryo and initiates the synthesis and secretion of proteases, α-amylases and cell wall degrading enzymes. B-Amylase which is already present in bound form in the endosperm is also activated. The majority hydrolytic enzymes increase during the 4-5 day germination period and continue through the early stages of kilning. Activity is eventually halted and the amount of activity retained for mashing depends on the enzyme type and manner in which kilning is conducted (Ullrich, 2011).

2.9. Usage of barley

The most important uses of barley are in the malting and brewing industry for beer and whiskey production, animal feed and human food. In Canada, about 83% of produced barley is used as livestock feed whereas 12% and 5% are destined for malting and other purposes, respectively. There has been a higher demand for food and malting barley in the last five to ten years as a result of increased health awareness and flavorable market prices (Baik and Ullrich, 2008). Novel uses of high β-glucan barley in the nutraceutical industry has

emerged lately (Delaney *et al.,* 2003) and industrial applications of high-amylose barley starches are under investigation to diversify barley utilization (Ganeshan *et al.,* 2008).

2.9.1. Nutrition and human health

About 2% of the global barley production is used for food (Baik and Ullrich, 2008). The preferred barley for food use is clean, thin-hulled, bright yellow-white, plump, medium-hard anduniform in size. A few two-row and six-row hulless genotypes with a minimal cleaning requirement meet these specifications. Dehulled, polished and milled barley is often used in porridge and soups, and as a substitute for rice incertain Asian countries e.g. Iran. Also a substantial amount of barley is used in baked foods such as breads, grits, noodle and pilaf in India and surrounding countries. Barley-based foods provide several positive effects on the human digestive system.Consumption of barley increases bulk and reduce transit time of fecal matter, which is associated with a lower frequency of hemorrhoids and colon cancer (Tsai *et al.,* 2004). Fermentation of barley's insoluble dietary fiber in large intestine produces short-chain fatty acids such as butyric acid that help to maintain a healthy colon (Behall *et al.,* 2004). Other fermentation products such as propionic and acetic acids provide fuel for liver and muscle cells (Liu, 2004). Propionic acid is also known to inhibit HMG-CoA reductase involved in cholesterol biosynthesis in liver (Erkkila *et al.,* 2005), thus lowering blood cholesterol levels. One of the important dietary fibers produced by barley is the soluble glucan polymer β-glucan (1→3, 1→4)-β-D-glucan. The concentration of β-glucan in barley grains is normally5% (but genotypes producing waxy or high-amylose starch generallyhave a higher concentration of β-glucan and dietary fiber (Izydorczyk *et al.,* 2000). The presence of β-glucan in diets increases the viscosity of foods during digestion leading to lower glucose

absorption and reduced blood glucose level measured as glycemic index (GI) (Jenkins *et al.,*2003). Foods with a low GI is preferred to decrease the risk of diabetes in humans. Beta-glucan also has a positive effect on cholesterol levels, as the fiber absorbs and removes bile acids produced from cholesterol in the liver. The absorption of bile acids triggers the liver to produce more bile acids from cholesterol (Brennan, 2005) and the net effect is a reduction in blood cholesterol levels (Behall, 2004). Barley fiber is also a good source of niacin, B-vitamin that reduces platelet aggregations that cause blood clots and lowers the levels of total cholesterol, lipoprotein and free radicals which oxidize low-density lipoprotein cholesterol. Thus, niacinprotects against cardiovascular diseases (Jood and Kalra, 2001). Consumption of food with 21g fiber per day have been suggested to lower the chances of coronary heart and cardiovascular diseases by 12-15% and 10-11%, respectively (Jensen *et al.,* 2004). As various health claims are associated with barley grain consumption, future barley based food products are aimed at regulation of blood sugar levels in diabetics, reducing cholesterol and lowering the incidence of heart disease. Besides low GI foods being desirable for diabetics, they are also beneficial for athletes requiring a slow release of glucose into the blood.

2.9.2. Malting and brewing

About 10% of barley produced worldwide is used to make malt for brewing beer. The malting cultivars include hulled, hulless, two-row and six-row varieties, but the hulled barley is preferred as hulls contributes to flavor and aids filtering during the brewing process (Gunkel *et al.,* 2002). Malting barley varieties are generally developed for a specific market e.g. domestic brewing or for export. Three-quarters of the area seeded to barley in Canada is occupied by two row malting barley, whereas barley growers in the US prefer six-row white aleurone

varieties.The six-row malting barley produced in Canada contributes 5% to the global barley trade and is mainly exported to the US market. The physical, chemical and biochemical properties of barley grain can have a large impact on the malting process and quality of beer. Kernel physical characteristics such as germination percentage, germ growth, kernel maturity, size, amount of seed-borne diseases and frost damage are factors that affect malting. The amount of grain starch, protein, β-glucan andtheir interactions during grain filling affect grain hardiness with effects on the yield of malt extract (Psota *et al.,* 2007). The alpha amylase level is another factor that determines the amount of malt extract. Preferred malting barley varieties are generally soft (Gupta *et al.,* 2010) with protein levels ranging from 10.5% to 13.0% for six-row types and 10.5% to 12.5% for two-row varieties. Barley with high protein concentration (> 15%) is not used for malting as it requires a long steeping time, has erratic germination and produces low malt extract (Swanston and Molina-Cano, 2001). Discolored barley grain is also unsuitable for maltingdue to undesirable flavors produced in beer by the breakdown of phenolics (Mussatto *et al.,* 2006). A successful sustenance of malting barley export market demands proper selection of cultivars with appropriate malting characteristics.

2.10. Fermentation

Fermentation is one of the oldest biotechnologies for the production of food products with desirable properties such as extended shelf-life and good organoleptic properties (Smid and Hugenholtz, 2010). Finished fermented foods usually have an improved microbial stability and safety and some can be stored even at ambient temperatures. Furthermore, there are several examples of fermentation processes which lead to an increase in nutritional value or digestibility (Jägerstad *et al.,* 2005) of food raw materials. Finally, food

fermentation processes also deliver products with increased palatability for consumers. All these arguments have boosted the interest to explore natural food fermentation processes and more precisely to link the diversity of the community of fermenting microbes and their properties to the energetics of the process and to product quality. From a biochemical point of view, fermentation is a metabolic process of deriving energy from organic compounds without the involvement of an exogenous oxidizing agent. Fermentation plays different roles in food processing. Major roles attributed to fermentation are: (1) Preservation of food through formation of inhibitory metabolites such as organic acid (lactic acid, acetic acid, formic acid, and propionic acid), ethanol, carbon dioxide, diacetyl, reutrin, bacteriocins, etc., often in combination with decrease of water activity (by drying or use of salt) (Gaggia *et al.,* 2011); (2) improving food safety through inhibition of pathogens (Adams and Nicolaides, 2008) or removal of toxic compounds (Poutanen *et al.,* 2009); (3) improving the nutritional value (Van Boekel *et al.,* 2010); and (4) organoleptic quality of the food (Sicard and Legras, 2011).

2.10.1. History of fermented foods

Fermentation as a food processing technique can be traced back to thousands of years. The history of fermented foods is lost in antiquity. It seems that the art of fermentation originated in the Indian Sub-continent, in the settlements that predate the great Indus Valley civilization (Prajapati and Nair, 2003). The art of cheese making was developed as far back as 8000 year ago in the fertile Crescent between Tigris and Euphrates rivers in Iraq, at a time when plants and animals were just being domesticated (Fox, 1993). Later, alcoholic fermentations involved in wine making and brewing are thought to have been developed during the period 4000–2000 BCE by the Egyptians and Sumerians. The Egyptians also

developed dough fermentations used in the production of leavened breads way back 4000–3500 BCE (Prajapati and Nair, 2003). However, the scientific rationale behind fermentation started with the identification of microorganisms in 1665 by van Leeuwenhoek and Hooks (Gest, 2004). Louis Pasteur revoked the "spontaneous generation theory" around 1859 AD by elegantly designed experimentation (Farley and Geison, 1974). The role of a sole bacterium "bacterium" lactis (*Lactococuuslactis*), in fermented milk was shown around 1877 by Sir John Lister (Santer, 2010). Fermentation, from the Latin word Fevere' was defined by Louis Pasteur as "la vie sans l'air" (life without air). Coincidentally, this was the time of the industrial revolution in Europe which resulted in large scale migration of populations from villages to larger cities. There was a dramatic shift from the food production for local communities to large scale food production, necessary to meet the requirements of expanding and more distant markets. This in turn led to the development of large scale fermentation processes for commercial production of fermented foods and alcoholic beverages, with the most widely used microorganisms including yeast for the production of beer, wine and spirits, and LAB for a variety of dairy, vegetable and meat fermentations (Ross *et al.,* 2002). Modern large scale production of fermented foods and beverages is dependant almost entirely on the use of defined strain starters, which have replaced the undefined strain mixture traditionally used for the manufacture of these products. This switch over to defined strains has meant that both culture performance and product quality and consistency have been dramatically improved, while it has also meant that a smaller number of strains are intensively used and relied upon by the food and beverage industries. This intensive use of specific starters has, however, some drawbacks and can lead to production problems resulting in unsatis factory strain performance. In the case of lactococcal fermentations, bacteriophage

proliferation can affect cheese starter performance (Klaenhammer and Fitzgerald, 1994). In 1928 CE, Rogers and Whittier discovered nisin produced by some LAB and demonstrated its antagonistic activity against other food-borne bacterial pathogens. In 2002, a complete list of microorganisms that can be used as safe microbial food culture in dairy industry has been released by the International Dairy Federation (IDF) (Mogensen *et al.,* 2002). The "2002 IDF inventory" has become a defacto reference for food cultures in practical use. In 2002, an updated inventory of microorganisms (bacteria, fungi, filamentous fungi and yeasts) used in food fermentations covering a wide range of food matrices was prepared by the members of IDF Task force (Bourdichon *et al.,* 2012).

2.10.2. Cereal fermentation

Recently, the fermentation of cereals has been extensively studied. Cereals are grown over 73% of the total world harvested area and account for 60% of world food production. Cereals have higher content of certain essential vitamins, prebiotic dietary fiber, and minerals than milk, but have lesser quantities of readily fermentable carbohydrates (Charalampopoulos *et al.,* 2002). The studies using cereal substrates and cultures as delivery vehicles for potentially probiotic lactic acid bacteria (Angelov *et al.,* 2005; Helland *et al.,* 2004). Kedia *et al.,* (2008) have also used mixed cultures for the fermentation of single cereals and cereal fractions. In the past, cereals were regarded as good substrates for the growth of probiotic strains (Charalampopoulos *et al.,* 2002) and cereal extracts were found to enhance acid and bile tolerance. The results showed that malt, wheat, and barley extracts were able to exhibit a significant protective effect on the viability of lactobacilli under acidic and bile conditions (Patel *et al.,* 2004). Cereal grains and their fractions contain many functional compounds, such as essential fatty acids, phytosterols, phenolic compounds or resistant starch, and

the consumption of whole grains has been associated with lower incidences of certain cancers and cardiovascular diseases (Truswell, 2002). Flavour is one of the most important characteristic in the sensory profile of a specific food and plays a decisive role in consumer acceptability. The analysis of volatile flavour components tends to be one of the main parameters to take into account for the development of a novel fermented food (McFeeters, 2004). Furthermore, the interaction between lactic acid bacteria and yeasts may affect the synthesis of volatile compounds. Unfortunately, the low content in proteins and essential amino acids (lysine), the low starch availability, and anti-nutrients (phytic acid, tannins and polyphenols) represent a drawback compared to milk and dairy products. However, fermentation could improve the quality of whole grain and cereal-based products (Gobbetti *et al.,* 2010). Due to the high levels of soluble dietary fibers (e.g., β-glucans), selenium and zinc, and antioxidant activity, oat and barley were used for making yogurt-like beverages. Based on nutritional and rheological properties, emmer flour was recently used for the manufacture of fermented beverages (Coda *et al.,* 2011).

2.11. Probiotics

2.11.1. Overview of probiotics

The most tried and tested manner in which the gut microbiota composition may be influenced is the use of live microbial dietary additions, as probiotics. In fact, the concept dates back as far as prebiblical ages. The first records of ingestion of live bacteria by humans are over 2,000 years old. However, at the beginning of this century probiotics were first put onto a scientific basis by the work of Metchnikoff (1908). He hypothesised that the normal gut microflora could exert adverse effects on the host and that consumption of 'soured milks' reversed this effect. The word ''probiotics'' was initially used as an anonym of the word

‘‘antibiotic’’. It is derived from Greek words pro and biotos and translated as ‘‘for life’’. The origin of the first use can be traced back to Kollath (1953), who used it to describe the restoration of the health of malnourished patients by different organic and inorganic supplements. Later, Vergin (1954) proposed that the microbial imbalance in the body caused by antibiotic treatment could have been restored by a probiotic rich diet; a suggestion cited by many as the first reference to probiotics as they are defined nowadays. Similarly, Kolb recognized detrimental effects of antibiotic therapy and proposed the prevention by probiotics (Vasiljevic and Shah, 2008) Later on, Lilly and Stillwell (1965) defined probiotics as “microorganisms promoting the growth of other microorganisms”. Later on, Lilly and Stillwell (1965) defined probiotics as “microorganisms promoting the growth of other microorganisms”. Following recommendations of a FAO/WHO (2002) working group on the evaluation of probiotics in food. Probiotics, are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Sanders, 2008; Schrezenmeir and De Vrese, 2001). The idea of health-promoting effects of LAB is by no means new, as Metchnikoff proposed that lactobacilli may fight against intestinal putrefaction and contribute to long life. Such microorganisms may not necessarily be constant inhabitants of the gut, but they should have a “beneficial effect on the general and health status of man and animal” (Bhadoria and Mahapatra, 2011).

2.11.2. Source of probiotics

Microbial strains serving as candidate probiotic are most commonly isolated from traditional fermented milk products. However, the isolation source varies between studies and regions and do have impact over functionality of isolates. Curd being consumed globally serves as the most preferred source. Other

fermented milk products including lassi, cheese(s) etc. are explored depending on their availability. Shelf life and functional aspects of fruits and vegetables are preserved and enhanced for long time by microbial fermentation. Fermented fruits and vegetables have a long history of use in human diet and are also associated with the several social aspects of different communities. Usually fermentation is carried out by the natural microflora of raw food products and progresses with succession by different microbes. Lactic acid fermentation increases shelf life, enhances nutritive value and flavours, and reduces toxicity (Swain *et al.,* 2014). Fermented fruits and vegetables can be used as a potential source of probiotics as they include wide spectra of LABs such as *Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus pentosus, Leuconostoc fallax, Leuconostoc mesenteroides* (Swain *et al.,* 2014). Microbial composition varies between regions, environmental conditions and type of fermentation. Although milk, food and vegetables are explored a lot for isolation of probiotic strains, researchers believe that strains with human origin may survive better during human gastric transit compared to those of non-human origin (Ranadheera *et al.*, 2014). Keeping this in mind, healthy human infant's faecal sample, healthy adult faecal samples and human breast milk samples are explored for selection of strains with rich probiotic potential.

Gastrointestinal tract of human have been inhabited by a variety of micro-organisms. Physiol*ogi*cal balance of this microbiota is greatly influenced by intestinal environment. Among the numerous intestinal bacteria that beneficially affect the host intestine, some could be recognized as probiotics (Ishibashi and Yamazaki, 2001). More than eighty species of microorganisms have been recorded as probiotics from various sources. Among them, the most used

Lactobacillussp. is present in raw milk, dairy products as well as infant, children and adult faeces (Coeuret *et al.,* 2003). The representative species of probiotics *viz., Lactobacillus* and *Bifidobacterium* include *Lactobacillus acdophilus, L. plantarum, L. johnsonii, L. gasseri, L. casei, L. rhamnosus, Bifidobacterium longum, B. breve, B. infantis, B. thermophillus, B. pseudolongum* and others in addition to these dairy product comprising *L. bulgaricus, Streptococcus thermophilus* and *Leuconostoc* could be used as probiotics Table 1.

Table 1. Microorganisms considered as probiotics

***Lactobacillus* species**	***Bifidobacterium* species**	**Other lactic acid bacteria**	**Non -lactic acid bacteria**
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*Bacillus cereus var. toyoi*
L. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli strai nissle*
L. casei	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freudenreichii*
L. crispatus	*B. breve*	*Leuconstoc mesenteroides*	*Saccharomyces cerevisiae*
L. delbrueckii	*B. infantis*	*Pediococcus acidilactici*	*Saccharomyces boulardii subsp*
L. gallinarum	*B. lactis*	*Sporolactobacillus inulinus*	*. bulgaricus*
L. gasseri	*B. longum*	*Streptococcus thermophilus*	
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

Source: Prado (2008)

2.11.3. Criteria of selection of appropriate probiotic

Different aspects have to be considered in probiotic selection Safety criteria for any successful probiotic have been defined in several reviews (Adams *et al.,* 2002) include the following specifications:

- Strains use is preferably of human origin.
- They are isolated from healthy human GI tract.
- They have a history of being non-pathogenic.
- They have no history of association with diseases such as infective endocarditis or GI disorders.
- They do not deconjugate bile salts (bile salt deconjucation or dehydroxylation would be a negative trait in the small bowel (Marteau *et al*., 2002).
- They do not carry transmissible antibiotic resistance genes.

While in selecting a preferable probiotic strain several aspects of functionality have to be considered:

- Acid tolerance and tolerance to human gastric juice.
- Bile tolerance (an important property for survival in the small bowel).
- Adherence to epithelial surfaces and persistence in the human GI-tract.
- Immuno stimulation, but no pro-inflammatory effect.
- Antagonistic activity against pathogens such as *Helicobacter pylori*, *Salmonella sp., Listeria monocytogenes* and *Clostridium difficile*.
- Antimutagenic and anticarcinogenic properties.

Feeding trials with different probiotic strains have shown that the probiotic strain usually disappears from the GI-tract within a couple of weeks after the ingestion is discontinued (Donnet-Hughes *et al.,* 1999). The role of the probiotic

persistence in the human GI-tract has therefore been questioned. However, even temporary persistence, which has been noted for several ingested probiotic strains, may enhance their chances for beneficial functions in the GI-tract, and is therefore considered a desirable trait. Necessary safety and functional criteria the aspects related to probiotic production and processing are also of utmost importance, such as:

1- Good sensory properties.

2- Phage resistance.

3- Viability during processing.

4- Stability in the product and during storage.

Good viability and activity of probiotics are considered prerequisites for optimal functionality. However, several studies have shown that non-viable probiotics can have beneficial effects such as immune modulation and carcinogen binding in the host (Salminen *et al*., 1999). Thus, for certain probiotic strains it might be sufficient that they grow well during initial production steps (to obtain high enough numbers in the product) but they do not necessarily need to retain good viability during storage.

2.11.4. Probiotics health benefits

Probiotic research suggests a range of potential health benefits to the host organism. The potential effects can only be attributed to tested strains but not to the whole group of probiotics. Probiotics have shown to provide a diverse variety of health benefits to human, animal, and plans. However, viability of the microorganisms throughout the processing and storage play an important role in transferring the claimed health effects. Therefore, the health benefits must be documented with the specific strain and specific dosage (Guarner *et al.,* 2009).

2.11.4.1. Human health

Probiotics display numerous health benefits beyond providing basic nutritional value (FAO/WHO, 2001). Some of these benefits have been well documented and established while the others have shown a promising potential in animal models, with human studies required to substantiate these claims (Vasiljevic and Shah, 2008). Health benefits of probiotic bacteria are very strain specific; therefore, there is no universal strain that would provide all proposed benefits and not all strains of the same species are effective against defined health conditions (Vasiljevic and Shah, 2008). Probiotics have been used in fermented food products for centuries. However, nowadays it has been claimed that probiotics can serve a dual function by their potentially importing health benefits. The health benefit of fermented foods may be further enhanced by supplementation of *Lactobacillus* and *Bifidobacterium* species (Shah, 2000). *L. acidophilus*, *Bifidobacterium spp*. and *L. casei* species are the most used probiotic cultures with established human health in dairy products, whereas the yeast *Saccharomyces cerevisiae* and some *E. coli* and *Bacillus* species are also used as probiotics (de Vrese and Schrezenmeir, 2008). Several studies have documented probiotic effects on a variety of gastrointestinal and extraintestinal disorders, including prevention and alleviation symptoms of traveler's diarrhea and antibiotic associated diarrhea, inflammatory bowel disease (Marteau *et al.,* 2002), lactose intolerance(de Vrese *et al.,* 2001), protection against intestinal infections(Reid *et al.,* 2001), and irritable bowel syndrome. Some probiotics have also been investigated in relation to reducing prevalence of atopic eczema later in life (Gueimonde *et al.,* 2006), vaginal infections, and immune enhancement (Isolauri *et al.,* 2001), contributing to the inactivation of pathogens in the gut, rheumatoid arthritis, improving the immune response of in healthy elderly people(Ibrahim *et al.,* 2010) and liver cirrhosis. In addition, probiotics are

intended to assist the body's naturally occurring gut microbiota. Some probiotic preparations have been used to prevent diarrhea caused by antibiotics, or as part of the treatment for Antibiotic related symbiosis. Although there is some clinical evidence for the role of probiotics in lowering cholesterol but the results are conflicting. Probiotics have a promising inhibitory effect on oral pathogens especially in childhood but this may not necessarily lead to improved oral health (Twetman and Stecksen, 2008).

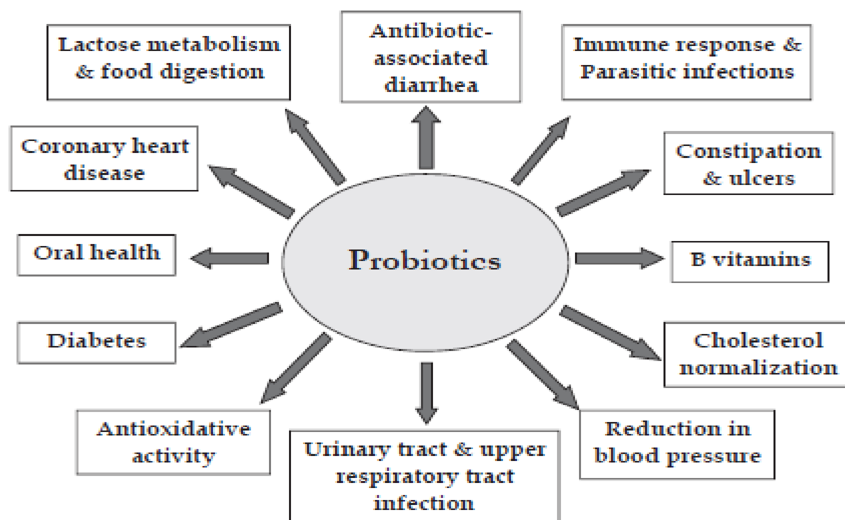

Figure 2.Probiotics health benefits

Antigenotoxicity, antimutagenicity and anticarcinogenicity are important potential functional properties of probiotics, which have been reported recently. Observational data suggest that consumption of fermented dairy products is associated with a lower prevalence of colon cancer, which is suggested that probiotics are capable of decreasing the risk of cancer by inhibition of carcinogens and pro carcinogens, inhibition of bacteria capable of converting procarcinogens to carcinogens (Vasiljevic and Shah, 2008).

2.11.5. Applications of probiotics into foods

Today an increase in knowledge of functional foods has led to develop foods with benefits health beyond adequate nutrition. The last 20 years have shown an increased interest among consumers in functional food including those containing probiotics. The presence of probiotics in commercial food products has been claimed for certain health benefits. This has led to industries focusing on different applications of probiotics in food products and creating a new generation of 'probiotic health' foods (Stanton *et al.,* 2003).

2.11.5.1. Dairy-based probiotic foods

Milk and its products is good vehicle of probiotic strains due to its inherent properties and due to the fact that most milk and milk products are stored at refrigerated temperatures. Probiotics can be found in a wide variety of commercial dairy products including sour and fresh milk, yogurt, cheese, etc. Dairy products play important role in delivering probiotic bacteria to human, as these products provide a suitable environment for probiotic bacteria that support their growth and viability. Several factors need to be addressed for applying probiotics in dairy products such as viability of probiotics in dairy (Phillips *et al.,* 2006), the physical, chemical and organoleptic properties of final products (Akin and Kirmaci, 2007), the probiotic health effect (Parvez *et al.,* 2006), and the regulations and labelling issues (Özer and Kirmaci, 2010).

2.11.5.2. Non-dairy based probiotic products

Dairy products are the main carriers of probiotic bacteria to human, as these products provide a suitable environment for probiotic bacteria that support their growth and viability. However, with an increase in the consumer vegetarianism throughout the developed countries, there is also a demand for the vegetarian probiotic products. Granato and others have overview of functional food

development, emphasizing non-dairy foods that contain probiotic bacteria strains (Granato *et al.,* 2010). From their review, some non-dairy probiotic products recently developed are shown in Table 2.

Table 2. Some non-dairy probiotic products recently developed

Category	Product
Fruit and vegetable based	Vegetable-based drinks Fermented banana pulp Fermented banana Beets-based drink Tomato-based drink Many dried fruits Green coconut water Peanut milk Cranberry, pineapple, and orange juices Ginger juice Grape and passion fruit juices Cabbage juice Carrot juice Noni juice Onion Probiotic banana puree Non-fermented fruit juice beverages
	Cereal-based puddings Rice-based yogurt Oat-based drink Oat-based products

Cereal based	Yosa (oat-bran pudding) Mahewu (fermented maize beverage) Maize-based beverage Wheat, rye, millet, maize, and other cereal fermented probiotic beverages Malt-based drink Boza (fermented cereals) Millet or sorghum flour fermented probiotic beverage
Other nondairy foods	Starch-saccharified probiotic drink Probiotic cassava-flour product Meat products Dosa (rice and Bengal gram)

Source: Granato *et al.,* (2010)

2.12. *Bifidobacterium* as probiotics

Bifidobacteria as probiotic members of the genus *Bifidobacterium* are some of the most common organisms in the human intestinal tract. *B. bifidum*, *B. breve*, *B. longum* and *B. animalis* are commonly used for the production of fermented milks, in combination with other lactic acid bacteria. Tissier first described Bifidobacteria in 1900 from breast-fed infants and termed it as "Bacillus bifidus". It has been suggested that *Bifidobacterium* species are important in maintaining general health because they contribute to a beneficial microflora in the intestinal tract and that the diversity and number of *Bifidobacterium* species provide a marker for the stability of the human intestinal microflora (Tanaka, 1995). Oral administration of Bifidobacteria may be effective for the improvement of intestinal flora and intestinal environment, for the therapy of enteric and hepatic disorders, decrease of serum cholesterol levels, for stimulation of the immune

response, and possibly for the prevention of cancer and slowing the aging process (Russell *et al.,* 2011). Therefore, many attempts have been made to increase the number of *Bifidobacterium* cells in the intestinal tract by supplying certain Bifidobacterial strains and food ingredients that stimulate the growth of Bifidobacteria as food additives (Gibson and Roberfroid, 1995).

2.12.1. Taxonomy

Classification and taxonomy of *bifidobacteria* have been a source of controversy since their discovery more than a hundred years ago. In 1900, Tissier isolated Gram-positive, anaerobic bacteria with a either to unknown Y-shaped morphology from the faeces of breast-fed infants, which he termed *Bacillus bifidus* communis (lat. bifidus: cleft, divided; Tissier, 1900). Shortly after the introduction of the Lactobacillaceae in 1917, the Tissier strains were integrated in this new family by Holland (1920) and renamed to Lactobacillus bifidus. In 1924, Orla-Jensen suggested the new genus *Bifidobacterium* for the representatives of the species based on morphological, cultural, and biochemical investigations. However, the former nomenclature prevailed, and only fifity years later the bifidobacteria officially obtained their deserved status as a separate genus by the publication of the VIIIth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). This reclassification was the consequence of sugar fermentation studies of the discovery of the *bifidobacterial* hexose catabolism as well as of analyses of the genomic G+C content, in which bifidobacteria strongly differ from the lactic acid bacteria. Today, the genus *Bifidobacterium* is included in the newly established class Actinobacteria, the Gram-positive bacteria with a high G+C content in their genome (Stackebrandt *et al.,* 1997). Currently approximately 30 species are differentiated, but as the flood of new publications referring to this topic shows

(Sakata *et al.,* 2002). Bifidobacteria can shortly be characterised as Gram positive, non sporeforming, non -motile, catalase negative, fermentative rods exhibiting pleomorphism, the cells being short or long, irregular, often curved, bifid or multiple-branched. The G+C content of their DNA varies from 55 to 67% (Biavati *et al.,* 2001). They are generally regarded as strictly anaerobic, yet some species possess a considerable oxygen tolerance (Meile *et al.,* 1997). They grow optimally between 37 and 41°C at pH values of 6.5 to 7, with the exception of the newly described *B. thermacidophilum*, which is able to grow at up to 49.5°C and at pH 4 (Dong *et al.,* 2000). Along with *Bacteroides, Eubacterium, Clostridium, Lactobacillus, Fusobacterium, Peptococcus, Peptostreptococcus, Enterococcus, Staphylococcus, Veillonella,* and *Enterobacteriaceae* species, bifidobacteria belong to the predominant bacterial groups within the intestinal microflora of humans, representing up to 15% of the cultivable bacteria of this ecosystem in adults and being the main pioneer colonisers in newborns (Marteau *et al.,* 2002). *Bifidobacteria* are also widespread in the gastrointestinal tract of animals such as calves, lambs, pigs, chickens, rabbits, rats, and even honeybees. Besides, they can be isolated from the human vagina, from the human oral cavity, and from sewage. In general, *Bifidobacterium* species are specific for either humans or animals, whereby the occurrence of the same species in suckling calves and breast-fed infants is the exception (Biavati *et al.,* 2001).

2.12.2. Morphology

The group of bacteria that belong to the genus *Bifidobacterium* presents rod shape morphology, Gram positive, non -motile and non -spore forming. This group of rod shaped bacteria have an irregular shape that lenght on average between 2 and 5 μm and presents swelling ends with club or slice form and with one or more branches, but even more regular and cocci shape are not so rare. This type of

polymorphism is generally species-specific, so that even the kind of morphology can be used for identification of bacteria. However, even in one single species it is possible to have different cells with different shapes; this is particularly due to growth conditions. Stress conditions as low pH, extreme temperatures or nitrogen starvation can trigger morphological alterations of bacterial cells that swell. When *Bifidobacterium bifidum* var. pensylvanicus is cultivated in a media without Nacethylglucosamine, the main component of glycane, assumes a club shape. When bifidobacteria are cultivated in a poor medium cells present the typical "Y" shape, but as soon as some amino acids like, alanine, glutamic acid, serine ad aspartic acid, are added, cells tend to assume a rod shape. Probably, these amino acids are implicated in peptidoglycan formation so that this pleomorphic is due more to an altered synthesis than to degenerative process (Tannock, 2002).

2.12.3. Physiology

2.12.3.1. Temperatures and pH

The origin of the different species of *bifidobacteria* reflects the different optimal temperature needed for their growth. Species of human origin prefer a range of temperature between 37°C and 41°C and cannot stand temperatures higher than 45° C. In the most of the species of animal origin growth is possible even at more than 45°C, while it has never been demonstrate that these bacteria can live at temperature lower than 25°C. When bacteria are stored at 4°C, cultures are viable up to 1 month; when are harvested at -80° C, their full viability can be prolonged for several years, but when are lyophilised the cultures can be recovered even after 30 years. Bifidobacteria are acid tolerant microrganisms, but not acidophil, so they grow in medium with a pH range between 5 and 7 preferring a restrict pH

range between 6.5 and 7. Growth inhibition is obtained at pH lower than 5 and higher than 8 (Talwalkar and Kailasapathy, 2004)

2.12.3.2. Oxygen sensibility

Bifidobacteria are anaerobe microrganisms, but oxygen sensibility differs from different species and different strains. There are strains less strictly anaerobe and there are mutant strains facultative anaerobes. The mechanisms that are implicated in oxygen tolerance are not well known. Anyway, bifidobacteria are catalase negative microrganisms, but among the aero-tolerant species *B. indicum* and *B. asteroides* become catalase-positive if grown in the presence of oxygen, respectively with or without the addiction of hemin. It has been hypotheses that atmospheric oxygen can interfere by two different mechanisms: helping redox potential, in this case the increasing oxygen is not lethal but can stop the growth of some strains, or producing H_2O_2, inhibitor of fructose-6-phosphate phosphoketolase, the key enzyme for sugar metabolism in bifidobacteria. Oxygen tolerance can be interpreted as an eventual capacity to degrade hydrogen peroxide (due to the slightly catalase activity as shown for *B. indicum* and *B. asteroides*) or to prevent its formation (Scardovi, 1986).

2.12.3.3. Ecology of *Bifidobacterium* spp.

Habitat the group of species that has been isolated so far comes from different sources and different niches. Human origin bifidobacteria were mainly colon resident, in fact it is indicative that their presence is massively reported in fecal samples of healthy humans and in the newborns. Species have been isolated from the oral cavity (*B. denticolens, B. dentium and B. inopitatum*) and even from vagina (*B. bifidum, B. breve, B. catenulatum, B. infantis and B. longum*), they represent a part of a consortium formed by other lactic acid bacteria e.g. *Lactobacillus spp.*. Bifidobacteria of animal origin are mainly reported from

mammal source. Presence of these bacteria has been highlighted in fecal samples from: rat, dog, cattle, pig, chicken, rabbit, while three species has been isolated from bees intestine. Some species seem to be host-specific, for example *B.magnum*, *B. suis* and *B.pullorum* have been specifically isolated from rabbit, pig and chicken, respectively. *B.dentium* is constantly associated with dental caries. *B.minimum* and *B. subtile* have been isolated from sewage and these two new species are the sole reported to habits nonliving ecological niches.

2.13. *Bifidobacterium longum* BB536

Bifidobacteria are the major components of intestinal microflora in humans. As probiotic agents, bifidobacteria have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human disorders, such as constipation, colonic transit disorders, intestinal infections, colonic cancer, and allergic diseases (Borriello *et al.,* 2003).

Bifidobacterium longum BB536 was originally isolated from a healthy infant in 1969. BB536 was first commercially available in Japan in 1977, with the launch of Morinaga Bifidus Milk. At present, a large number of products ranging from dairy products to supplements have been marketed in Japan. Presently, BB536 is also broadly available in the European, USA, and Asian marketplaces. BB536 was characteristic for its high survivability in food applications and its high accessibility to the gastrointestinal tract. Lines of evidence including in *vitro*, in *vivo*, and clinical studies and consumption history have supported the safety of BB536. Accumulated data have also shown the health benefits for BB536 in various hosts.The species of bifidobacteria now referred to as Bifidobacterium longum contains three subspecies: longum, infantis, and suis which were originally categorized as separate species. Subsequent analyses of their sequences revealed a high degree of homology leading to their inclusion under one species,

B. longum (Sullivan, 2010). *B. longum* spp. infantis and longum have been isolated from the infant and adult intestine and several strains from both subspecies have been studied in the laboratory, as well as in clinical trials as probiotics (Underwood *et al.,* 2014).

2.14. Evaluation of *B. longum* BB536 *as* a probiotic microorganism

Despite the general safe use of bifidobacteria, some side effects in susceptible individuals are theoretically possible. In consideration of the potential adverse effects, FAO/WHO guidelines for the evaluation of microbes for probiotic use in foods have recommended testing for several parameters, including antibiotic resistance, metabolic activities (e.g., D-lactate production, bile salt deconjugation), toxin production, hemolytic activity, infectivity, side effects during human studies, and adverse incidents in consumers. These parameters have been examined for *B. longum* BB536. Data from in *vitro* studies, acute, chronic, and repeat dose animal studies, clinical studies involving healthy and unhealthy adults, or children, as well as a long historical consumption of almost 30 years have provided information that supports the safety of the use of *B. longum* BB536. For example (1) an evaluation on antibiotics, which demonstrated that BB536 is not an antibiotic-resistant strain, and which also reported that resistant gene was not found there; (2)strain BB536 was found to produce L-lactic acid predominantly, while the production of D-lactic acid was negligible; (3) strain BB536 was observed to possess a conjugated bile salt hydrolase that was able to deconjugate 80–95% of the selected bile salts seen in concurrent bacterial growth, with the only compounds produced being the deconjugated bile salts; (4) genomic analysis of BB536 failed to find any high homological sequences with amino acid sequences of known bacterial toxins that are listed in the Gene Bank database; and (5) tests of hemolytic activity of BB536

by using BL agar plates supplemented with horse blood indicated that *B. longum* BB536 does not have any hemolytic activity (Xiao *et al.,* 2007) . On the basis of these safety investigations, clinical observations, and the long use experiences within the food category, BB536 has been accepted as a GRAS strain for its intended use in the USA.

B. longum has anti-inflammatory properties that protect the cells lining your mucous membranes from toxins and help some of your immune cells to mature so they can function properly. This probiotic microbe is also present in breast milk, and is one of the first microbes to colonize the infant gut. It also has enzymes to digest proteins so that they don't putrefy in the colon. Putrefaction is not healthy for the colon (Yaeshima *et al.,* 1997).

Some of the conditions that *B. longum* has shown health benefits for are:

- Gastrointestinal upset.
- Antibiotic-associated diarrhea.
- Pathogen infections.
- Seasonal allergies.
- Possible weight maintenance.
- Bone health.
- Colon cancer prevention.
- Cholesterol-lowering.

It is able to ferment a wide variety of carbohydrates, including lactose and the sugars in cruciferous vegetables, dried beans and some cereal grains that humans cannot digest (Yaeshima *et al.,* 1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

3.1.1. Barley varieties

Two barley varieties (Bukur and Balady) used in this study were obtained from Agriculture Research Center, Hadhrmout (Yemen) and (Local 46) from Agriculture Research Corporation, Ministry of Agriculture, Shambat (Sudan). All barley varieties were manually cleaned and sorted by removing extraneous materials and damaged seeds, followed by winnowing removal dust. All varieties were packed in plastic containers till used.

3.1.2. Chemicals (analytical grade)

All chemicals (Analytical grade) and microbiological media were purchased from local company (Lab Tech, Khartoum North, Sudan).

3.2. Methods

3.2.1. Malting of barley

Malting of barley was carried out at Food Microbiology Laboratory, College of Agricultural Studies, Sudan University of Science and Technology (SUST). Cleaned barley grains were washed and soaked in distilled water at ratio of (1: 3 w/v), using glass beaker at 30ºC for 24hours. Then water was renewed every 12 h. The barley grains were spread on aluminum foil and incubated for 120 hours at 30°C with 12hours interval water spraying. At the end of germination period, the grains were dried in an oven at 55°C for 12hours, and the roots of the germinated barley were removed manually (Badau, 2004).

3.3. Analysis

3.3.1. Proximate analysis

3.3.1.1. Determination of moisture

Moisture was determined according to the modified method of AOAC (1990). Five grams of the sample was weighted in sensitive balance, after weighting the dishes was transferred to an oven (Kat-NR. 2851, Electrohelios, Sweden) at 105 ± 0.1˚C for 6 hours. Afterwards, the dish with sample was transferred to desiccators and allowed to cool at room temperature before reweighting. The moisture content was calculated according to the following formula:

$$\text{Moisture \%} = \frac{W1 - W2}{W1 - W} \times 100$$

Where:

W1 = Weight of the dish with the material before drying.

W2 = Weight of the dish with the material after drying.

W = Weight of the empty dish.

3.3.1.2. Determination of fat

Fat content was determined according to the official method of AOAC (1990). A sample of 5g was weighed into an extraction thimble and covered with cotton, and then extracted with hexane. The thimble containing the sample and a pre-dried weight extraction flask containing about 100 ml hexane was attached to the extraction unit. The extraction process was conducted for 16h. At the end of the extraction period, the flask was disconnected from the unit and the solvent was evaporated. Later, the flask with the remaining crude hexane extracted was put

in an oven (50– 60ºC),cooled to room temperature reweighted and the dried extract was registered as fat content.

$$\text{Crude fat}(\%) = \frac{\text{W2} - \text{W1}}{\text{Sample Weight}} \times 100$$

Where:

W1= Weight of the empty extraction flask.

W2= Weight of the extraction flask after the extraction process.

3.3.1.3. Determination of ash

The ash content of the sample was determined according to the AOAC (1990) method. Two grams of the sample were weighed into a clean dry porcelain crucible and placed in muffle furnace at 600˚C for 6 hours. The crucible was transferred to a desiccator; cooled to room temperature and the ash content was calculated as follows:

$$\text{Ash content}(\%) = \frac{\text{w1} - \text{w2}}{\text{Weight of sample}} \times 100$$

Where:

W1 = Weight of crucible with ash.

W2= Weight of empty crucible.

3.3.1.4. Determination of protein

The protein content was determined by Kjeldahl method according to the AOAC (1990) method as follow:

1. **Digestion**: Two grams of the different fermented products were weighed in a crucible and transferred to a digestion flask with two tablets catalyst (Mercury). 25 ml of concentrated sulphuric acid were added to the samples, the flask was

placed on the digestion apparatus, heated until the mixture was colour less. Then the flasks were allowed to cool at room temperature.

2. **Distillation**: Twenty five ml of boric acid and three drop of bromocresol green+ methyl red indicator were added to each receiving flask. The digested samples were transferred from the digestion flask to volumetric flask and the volume was completed to 100 ml by distilled water. The receiving flask was placed on the distillation rack with the tip of the condenser extended below the surface of the acid. Immediately 5 ml of the diluted samples were added from the funnel of the distillation apparatus, then 10 ml NaOH (40%) was gently added. The distillation was continued until the volume in the receiving flasks were 7 ml, then the flasks were removed from the distillator.

3. **Titration**: The samples in the receiving flasks were titrated against 0.1N HCL. The colour was changed from green to purple. The nitrogen content was calculated as follows:

$$\%N = \left[\frac{\text{ml HCL}\times \text{normality of HCL}(0.1)\times 0.014\times 100}{\text{Sample weight}}\right]$$

%Protein= %N ×6.25

Where:

N = Nitrogen content.

0.014=molecular weight of nitrogen/1000

3.3.1.5. Determination of crude fiber

Fiber was determined according to AOAC (1990) . Two g of defatted sample was placed into a conical flask containing 200ml of H_2SO_4 (0.26N). The flask was fitted with a condenser and allowed to boil for 30 minutes. At the end of the

digestion period, the flask was removed and the digest was filtered through a proclaim filter crucible (No.3). After that, the precipitate was repeatedly rinsed with distilled boiling water, followed by boiling in 200ml NaOH (0.23N) solution for 30 min under reflux condenser and the precipitate was filtered and rinsed with hot distilled water, 20 ml ethyl alcohol (96%) and 20ml diethyl ether. Finally, the crucible was dried at 105ºC until a constant weight was obtained and the difference in weight was considered a crude fiber.

Crude fiber % =

$$\frac{[(\text{Dry residue } + \text{ crucible(g)} - \text{ (ignited residue } + \text{crucible (g))}]}{\text{Weight of sample}} \times 100$$

3.3.1.6. Calculated carbohydrates

Carbohydrates were calculated by difference according to the following: A available Carbohydrates = 100% - [Moisture (%) + Protein (%) +Fat (%) +fiber (%) + Ash (%)].

3.4. Determination of sugar content

3.4.1. Total sugars

From the previous clear sample solution for determination of acidity, 50 ml was pipetted into a 250 ml conical flask and 5g citric acid and 50 ml distilled water was added slowly. Then, the mixture was gently boiled for 10 min to complete the inversion of sucrose and left to cool at room temperature. The solution was then transferred to 250 ml volumetric flask, neutralized with 20% NaOH solution in the presence of a few drops of phenolphthalein (NO. 6606 J. T Baker, Holland) until the color of the mixture disappeared and the sample was made up to volume before titration. Procedure: A volume of 10 ml of the mixture of Fehling's (A) and (B) solutions was pipetted into 250 ml conical flask. Then, sufficient amount

of the clarified sugar solution was added from a burette to reduce Fehling's solution in the conical flask. After that, the solution was boiled until a faint blue color is obtained. Then, a few drops of methylene blue indicator (s-d-Finechem Limited) were added to the Fehling's solution and titrated with sugar solution until brick-red color of the precipitate cuprous oxide was observed. Finally, the titer volume was recorded and the amount of inverted sugars was obtained from Lane and Eynon Table. The total sugars were calculated by using the following formulas:

Calculation: Total sugars {% DM} =

$$\frac{\text{(Inverted sugars (mg) x dilution factor)}}{\text{Titer x sample weight (g)x(100\% } - \text{ moisture \%)x1000}} \text{x100}$$

3.4.2. Reducing sugars

reducing sugars were determined according to Lane and Eynon titrometric method (AOAC, 1990). Ten grams of sample were weighed in volumetric flask. The volume of the solution was completed to 100 ml in conical flask. Burrete (50 ml) was filled with the prepared sugar solution. Ten millilitres of sugar solution was transferred into a conical flask containing 10 of ml Fehling's solution (5 ml of Fehling A: 6.928gm Cu $SO_4.5H_2O$ per 100ml distilled water) and 5 ml Fehling B: 34.6 sodium potassium tartrate and 10 gm NaOH per 100 ml distilled water), mixed well and then heated moderately to boiling point on an electrical hot plate heater. The liquid was kept boiling for about 2 minutes then 3 drops of methylene blue indicator (1%) was added. The titration was then completed by the addition of sugar solution drop by drop until the color of the indicator disappeared and red brick color appeared. The reducing sugar was calculated from the following equation according to, Schneider (1979).

$$\text{Total sugars (\%)} = \frac{\text{Fehlings soultion factor} \times 100 \times \text{dilution}}{\text{Volume of sample used}}$$

3.4.3. Non-reducing sugar

Non reducing sugars were determined according to the formula given below:

Non reducing sugars (%)= (Total sugars(%)- Reducing sugars(%)× 0.95

3.5. Determinationof minerals

Potassium (K), Sodium (Na) and calcium (Ca) were determined by flame photometer (Sherwood Flame Photometer i410, Sherwood Scientific Ltd. Cambridge, UK) according to procedure of AOAC (1990). The knob of flame photometer was adjusted to potassium, sodium and calcium respectively and reading was set to zero using deionized water. Blank solution was run and reading was again set to zero. Standard solution of each mineral was run and recorded the reading of flame photometer. The reading of potassium, sodium and calcium in each sample was taken by running the sample one by one. Standard solution was run after every sample .The standard curves were obtained by plotting absorbance values of standards against appropriate concentrations of these three elements. One gram of dried samples was subjected to wet digestion method as described by Richards, (1968). Then analysis was conducted through absorption spectrophotometer (Varian AA 240, Victoria, Australia) for determination of minerals (Mg and Fe) using standard curve. To determine phosphorus content in samples, colorimetric estimation method was used as described by Kitson and Mellon, (1944).

3.6. Phytochemical content

3.6.1. Phytate

Phytic acid was determined by the method of Haug and Lantzsch (1983).

Reagents

Phytate reference solution: Exactly 30.54 mg sodium phytate (5.5% water, purity and containing 12 Na/mole) was dissolved in 100 ml of 0.2 N HCl which gave a solution containing 200 µg phytic acid per ml. Ferric ammonium sulphate solution: Ferric ammonium sulphate (0.2g) was dissolved in 100 ml of 2N HCl and made the volume of 1000 ml with distilled water.

Bipyridine solution: Ten grams 2-2 pyridine and 10 ml thioglycollic acid were dissolved in distilled water and volume was made to 1000 ml. These solutions are stable for several months at room temperature.

Extraction

Finely ground sample (0.5 g) was extracted with 25 ml of 0.2 N HCl for 3 hours continuous shaking in a shaker. Thereafter, it was filtered through Whatman # 1 filter paper.

Estimation

An aliquot (0.5 ml) of the above extract was pipetted into a test tube fitted with a ground glass stopper. One ml of ferric ammonium sulphate was added. The tube was heated in a boiling water bath for 30 minutes. The contents of the tube were mixed and centrifuged at 3,000 rpm for 30 minutes. One ml of supernatant was transferred to another test tube and 1.5 ml bipyridine solution was added .The absorbance was measured at 519 nm against distilled water. For plotting a standard curve, different concentrations (0.2 to1.0 ml) of standard sodium phytate solution containing 40-200 µg phytic acid were taken and made to 1.4 ml with water O.D. of 0.342 corresponded to 80 µg phytic acid.

3.6.2. Polyphenols

Total polyphenols were extracted by the method of Singh and Jambunathan (1981). Defatted sample (500 mg) was refluxed with 50 ml methanol containing

one per cent HCl for four hours. The extract was concentrated by evaporating on a hot water bath and brought its volume to 25 ml with methanolic–HCl. The amount of polyphenolic compounds was estimated as tannic acid equivalent according to Folin-Danis procedure.

Reagents

i) Folin-Denis reagent: To 750 ml water, 100 g sodium tungstate, 20 g phosphomolybdic acid and 50 ml phosphoric acid were added and heated and then refluxed for 2 hours. It was cooled and diluted to one litre.

ii) Tannic acid (stock solution): 100 mg of tannic acid was dissolved in water and made upto one litre. In order to have working standard solution, 20 ml stock solution was further diluted to 100 ml with water.

iii) Saturated aqueous sodium carbonate solution: Dissolved 350g sodium carbonate in one litre hot distilled water at 70°C to 80°C, cooled and filtered through glass wool.

Procedure

Test solution (1.5 ml) was diluted with distilled water to 8.5 ml in a graduated test tube. After thorough mixing, added 0.5 ml Folin-Denis reagent and the tubes were well shaken. Exactly after 3 minutes, one ml of saturated sodium carbonate solution was added and the tubes were thoroughly shaken again. After an hour, the absorbance was read at 725 nm on UV- VIS Spectrophotometer 118 using a suitable blank. If the solution was cloudy or precipitates appeared, it was centrifuged before readings were taken. A standard curve was plotted by taking 0.5 ml to 4.0 ml working tannic standard solution containing 10 to 80 µg tannic acid.

$$\text{Polyphenols (mg/100g)} = \frac{\text{M} \times \text{V} \times 100}{\text{W} \times \text{V1} \times 1000}$$

Where,

M = Concentration of extract elute obtained from graph

V = Volume made of extract (ml)

W = Weight (g) of the sample

V1 = Volume of extract aliquot taken (ml)

3.6.3. Tannin content

Tannin content of the each sample was determined according to the modified Vanillin-HCl methanol method as described by Price *et al.,* (1980). The Vanillin-HCl reagent was freshly prepared by mixing equal volumes of 8% concentrated HCl in methanol and 1% vanillin in methanol. For determination of tannin content, extracts were obtained by mixing 2 g of samples (1 g in case of barley flour) in 20 ml acidified methanol (1% concentrated HCl). The suspension was vigorously stirred for 90 minutes using magnetic stirrer and centrifuged at 3000×g for 10 min. 1.0 ml of the supernatant was pipetted into a test tube containing 5ml of Vanillin-HCl reagent. Absorbance at 450 nm recorded using spectrophotometer, after 30 minutes incubation at 30°C. A sample blank was prepared by using 1.0 ml of acidified methanol in place of sample extract and processed subsequently in similar manner. A set of catechin hydrate standard solutions ranging from 0 to 120 ml/l was prepared using methanol as a solvent to draw calibration curve and results were expressed in terms of catechin equivalent (mg/g).

Tannin content was expressed as catechin

$$\text{Tannin}(\%) = \frac{(C * 10) * 100}{200}$$

Where:

C = Concentration of corresponding to the optical density,

10 = volumes of the extract (ml)

and 200g = sample weight.

3.6.4. Flavonoid

Flavonoid content was estimated by the method described by Zhishen *et al.* (1999). About 0.1 ml of aliquot from each extraction was taken and volume was made up to 5 ml with distilled water. At 0 time, 5 % $NaNO_2$ (0.3 ml) was added, after 5 min, 10 % $AlCl_3$ (0.6 ml), and at the 6th min, 1 M NaOH (2 ml) solution was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Standard series was prepared using known concentration of rutin, final volume as made up to 5 ml with distilled water and there after treated in similar way as for sample.

3.7. Fermentation process

3.7.1. Preparation of fermentation inoculums

Bifidobacterium longum BB536 was obtained from the stock culture of Microbiology Laboratory (Department of Food Science Technology, Collage of Agriculture Studies, SUST). The strain was maintained at -20˚C in 20% glycerol solution. Stock culture was prepared by activation of the strain in skim milk, incubation anaerobically at 37˚C for 24h. The obtained culture was reactivated again under the same conditions to prepare enough stock for the experiment. The working culture was prepared by twice success transformation in 10% sterilize skim milk (121˚C for 15 mins) and incubation at 37˚C for 24h.

3.7.2. Preparation of barley beverage

Barley beverage was prepared by the same method that reported by (kabeir *et al.,* 2009) with slight modification. The malted barley flours (10%) was mixed with water in a ratio of (1:4 w/v) and then blended for 5 mins at blended medium

speed. The slurry, formed was filtered by using a double layer cheese cloth, obtain the beverages.

3.7.3. Fermentation medium

The control medium formulated from 10% re-constitutedskim milk in addition other three barley variety beverages included (Bukur, Balady and Local 46). All Formulated medium were sterilized (121ºC for 15 min) and inoculated with 3% active of *B.longum* BB536 culture. After that the mixture was incubated anaerobically at 37º C for 24h to obtain the fermented beverage.

3.7.4. Enumeration of *Bifidobacterium longum* BB536

The enumeration of *B. longum* BB536 of different fermented beverages was attained using the plate count technique with De Man Rogosa Sharpe agar(MRS) medium. The fermented samples were drawn at the initial time (0h) and every 6h intervals during fermentation. One ml of fermented beverage was used to make serial dilution in 9 ml peptone water, followed by plating on De Man Rogosa Sharpe agar (MRS) supplement with 0.05% L- cystiene. The plates were incubated anaerobically at 37˚C for 48 h. The growth was calculated as colony forming unit per ml (cfu/ml).

3.7.5. The storage of the fermented products

The fermented beverages were held at refrigerator temperature 4 ºC for three weeks. Throughout the storage time, the viable counts of *Bifidobacterium longum* BB536, pH, titrable acidity, TSS, moisture and sugar content of the fermented beverages were determined. Analysis of samples were carried out at the initial time (maximum growth), one week, two week and three weeks intervals.

3.8. Physico-chemical analysis

3.8.1. Determination of titratable acidity

The titratable acidity (TA) of the different fermented beverages was determined according to AOAC (1990). Ten ml of sample were weighted into a conical flask. Distilled water was added until the volume in the flask was 150 ml. The sample was then vigorously agitated and filtered. Twenty-five milliliters of the filtrate were pipette into a porcelain dish, five drops of phenolphthalein added, and the sample was titrated against 0.1N NaOH till a fain pink color that lasted for at least 30 seconds was obtained. Acidity of different beverage samples was calculated from the following equation:

$$\text{Titratable} = \frac{\text{N} \times \text{T} \times 0.9}{Weight\ of\ Sample} \times 100$$

Were:

N= Normality of NaOH.

T= Titre value of NaOH

0. 9 = Factor of lactic acid.

3.8.2. Determination of total soluble solids (TSS)

Total soluble solids (TSS) of fermented beverages were determined at room temperature using digital Refractometer with degree Brix° scale 0-100 according to AOAC (1990).

3.8.3. Determination of pH value

The pH value of the different fermented beverages was determined using a pH-meter (model HI 8521 microprocessor bench PH/MV/C° meter. Romania). Two standard buffer solution of pH 4.00 and 7.00 were used for calibration of the pH

meter at room temperature. The pH meter was allowed to stabilize for one minute and then the pH of the fermented samples was directly measured.

3.9. Sensory properties of different fermented barley beverages

Different fermented barley beverage samples were subjected to sensory evaluation using semi-trained panelists according to Meilgaard *et al*. (1999). The samples were assessed for texture, color, flavor, and overall acceptability.

3.10. Safety of different fermented barley variety beverages at refrigeration

3.10.1. Preparation of equipment and media

3.10.1.1. Sterilization of glassware and metal tools

According to Harrigan and MacCance (1998) glassware was washed thoroughly with distilled water and left to dry, and then they were sterilized in a hot forced air oven at 160ºC for at least three hours. Inoculation wires and loops were sterilized by direct flaming to red-hot and other metal instruments such as spatulas and forceps were sterilized by flaming.

3.10.1.2. Sterilization of media

Media were sterilized by using an autoclave at 121ºC and 15 lb/in_2 for15 minutes; media containing sugar were sterilized using an autoclave at 110ºC for 10 minutes.

3.10.1.3. Preparation of the samples

Different types of fermented barley beverage and skim milk samples stored under the similar conditions were taken in sterile plastic containers. Fifty grams of samples were taken for microbiological examination. Samples were taken aseptically from containers. Ten grams of samples were added to 90 ml of distilled water in a flask and shaken well to complete mixing after that to make 10^{-2} dilution then 1 ml from the above mentioned dilution (10^{-1}) was aseptically

transferred to 9 ml sterile peptone water. This procedure was repeated to make serial dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. from suitable dilutions, 1 ml was transferred to Petri-dishes followed by pouring of 18-20 ml the culture medium aseptically to each Petri- dish , mixed gently , left to solidity and incubated in an inverted position (Houghtby *et al*., 1993).

3.10.2. Enumeration of viable cell of *B. longum* BB536

MRS medium was used to enumerate *B. longum* BB536 of different fermented barley beverage using the plate count technique. Samples were drawn at one day and every week intervals during storages (21 days). One gram of beverage fermented was diluted in 9 ml peptone water, followed by plating on Rogosa agar (MRS) supplement with 0.05% L- cystiene. The plates were incubated an aerobically at 37 ˚C for 48 h. The growth was calculated as colony forming unit per g (cfu/g).

Colony counters where:

Colony forming units/gm (cfu/gm) = Total number of colonies in the dilution multiplied by the reciprocal of dilution (Houghtby *et al*., 1993).

3.10.3. Total bacterial count (TBC)

Using nutrient agar medium Total bacterial count was determined according to the method by APHA, (1992).

3.10.3.1. Preparation of the media

The medium was prepared according to manufacturer's instructions by dissolving 23.5 gram of powder to1000 ml of distilled water, heated to boiling point and then sterilized in an autoclave at 121 ºC for 15 minutes.

3.10.3.2. Plating

From each selected dilution1 ml was transferred into sterile Petri dishes followed by addition of 15 – 18 ml melted, cooled (45 °C) nutrient agar and mixing thoroughly by rotating the dishes first in one direction and then in the opposite direction. When medium was solidified, the dishes were incubated in an inverted position at 35 ± 2 °C for 24 hours.

3.10.3.3. Counting

Plates contain 25 – 250 colony were selected and counted using colony counter. The number of colony forming units (CFU) in the dilution was obtained by multiplying the reciprocal of the dilution.

3.10.4. *E.coli* counts

The count was performed according to (APHA, 1992). using MaConkey agar media and Eosin Methylene Blue agar (EMB) for identification.

3.10.4.1. Preparation of the media

The manufacturer's instructions were followed by dissolving 55 grams of powder to 1000 ml of distilled water, heated to boiling point and sterilized in an autoclave at 121 °C for 15 minutes.

3.10.4.2. Plating and counting

One ml amounts of each sample dilutions were streaked in dried plate of Maconkey agar media. The culture was incubated at 35 °C for 24 hours, and then colonies were used for further confirmation of the presence of *E. coli* by streaking a loopful from each colony on Eosin methylene blue agar (EMB) for identification of colonies which show brilliant green, the characteristic features of growth of *E. Coli* in the medium.

3.10.5. S*taphylococcus spp.* counts

The count was achieved according to method by Christen *et al*. (1992) using Mannitol salt agar.

3.10.5.1. Preparation of the media

The manufacturer's instructions were followed by dissolving 111 grams of powder to 1000 ml of distilled water, heated to boiling point and sterilized in an autoclave at 121 ºC for 15 minutes (Christen *et al*., 1992).

3.10.5.2. Plating and counting

One ml quantities of each sample dilutions 10^2 - 10^6 was transferred into sterile Petri dishes followed by addition of 15–18 ml melted media, cooled (45 ºC) petridishes with Mannitol salt agar was mixed thoroughly by rotating the dishes first in one direction and then in the opposite direction. When medium was solidified. The culture was incubated at 35 ± 2°C for 24hours where colonies of *staphylococcus* were recognized by bright yellow zones formation in Mannitol salt agar. And then colonies were counted by colony counter.

3.10.6. Yeast and molds count

The yeast and molds count were determined according to Harrigan and McCance (1998).

3.10.6.1. Preparation of the media

Media was prepared according to manufacture instructions by suspending 39 grams of Potatoes dextrose agar in one liter distilled water and boiled until it dissolved completely, then it was sterilized by autoclaving at 121ºC for 15minuts.

3.10.6.2. Plating and counting

One ml from suitable dilutions was transferred into sterile Petri dishes followed by addition of 15 – 18 ml potato dextrose agar. The plates were incubated at 25°C for up to 72 hours. Then colonies were counted by colony counter.

3.10.7. *Salmonella*

The count was achieved according to method by Liong and Shah, (2006) using Brilliant green agar

3.10.7.1. Preparation of the media

Media was used according to manufacture instructions by suspending 58.09 grams of Brilliant green agar in one of liter distilled water , boiled until dissolved completely and then it was sterilized by autoclaving at 121ºC for 15 minuts.

3.10.7.2. Plating

A One ml quantity of each sample dilutions was transferred into sterile Petri dishes followed by addition of 15 – 18 ml melted media, cooled to 45ºC. Petri dishes with Brilliant green agar was mixed thoroughly by rotating the dishes first in one direction and then in the opposite direction. When media was solidified. The culture was incubated at 35 ± 2 °C for 24hours.

3.10.7.3. Counting

Plates contain 30–300 colony were selected and counted using colony counter. The number of colony forming units (CFU) in the dilution was obtained by multiplying the reciprocal of the dilution.

3.11. In *vivo* rat's experimental tests

3.11.1. Animals

Six weeks old male albino rats with an average initial weight of (95g±10) were purchased from College of Pharmacy, Ahfad University for Women (Khartoum, Sudan).They were housed six per cage. A 12 h light dark cycle and a controlled atmosphere (22.11±2.36°C) were maintained throughout the study. After fifteen days acclimatization period under experimental condition, rats were randomly assigned into five different groups (n = 6) at random and treated for thirty days. During the thirty day strial, the rats were offered water and fed adlibitumon the

all groups. The bed covers in cages was changed twice a week. Individual body weight of every rat used to be measured at ten days intervals.

3.11.2. Chemical and apparatus

All chemicals and kits were purchase from BioSystems (Spain) which were provided by local company in Khartoum (Labtech). Test tube, (K3 EDTA) and sterile container were obtained on local company in Khartoum (LABTECH).

3.11.3. Experimental design and feed groups

All experimental design was shown in table (3).

Table 3. The experimental rat groups and their diets

Group	Experimental diets (per 6 rats)
Control(C)	Normal diet + sterile water+3ml orally daily distilled water
Treatment M	Normal diet + sterile water+3ml orally daily strain BB536 fermented re-constituted skim milk
Treatment B	Normal diet + sterile water+3ml orally daily of strain BB536 fermented Bukur barley beverage
Treatment C	Normal diet + sterile water+ 3ml orally daily of strain BB536 fermented Balady barley beverage
Treatment D	Normal diet + sterile water+ 3ml orally daily of strain BB536 fermented Local 46 barley beverage

3.11.4. Clinical chemistry of blood of different treated rat groups

3.11.4.1. Method of blood collections

Blood samples for three rats of each group were collected at the end of the experiment period (30 days)from vein plexus eye in sterile tubes containing EDTA and kept at 4 ºC for haematology analysis, after that the rats were sacrificed under anesthetize and the blood was collected in clean test tubes. Serum was separated by centrifugation at 15.000 rpm for 20min to investigate the biochemistry parameter.

3.11.4.2. Analysis of blood hematology

Blood samples were analyzed for complete blood profile including: red blood cell (RBC), white blood cell (WBC), platelet (PL), hemoglobin concentration (HGB), and leukocyte differential count (NEU, LYM, etc.), platelet count (PLT), hematocrit (HCT), red blood cell indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC). The measurements were performed by Hematology Analyzer (Mindry-BC 3000 plus, USA).

3.11.4.3. Analysis of blood biochemistry

3.11.4.3.1. Serum enzymes and minerals

Serum enzyme [aspartate transferase (AST), alanine transferase (ALT) and alkaline phosphatase (ALP)] activities and total protein (TP), albumin (Alb), globulin (Glob) content and uric acid of the blood were estimated according to methods based on the enzymatic colorimetric test method **,** Also(Na, Ca, K and P) were determined using kits method obtained from BioSystems (Spain).

3.11.4.3.2. Glucose

Measurement was based on the colorimetric end point test method.

Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine product.

Protocol

In the followings were performed:

	Blank	Standard	Sample
Sample	-	-	10 µL
Standard	-	10 µL	-
Enzyme reagent	1000 µL	1000 µL	1000 µL

The reaction mixture was mixed well and incubated for 5 minutes at 37°C. The absorbance of sample and standard was measured against reagent blank at 505 nm. The concentration of the standard was then multiplied by the product of the sample absorbance/standard absorbance.

3.11.4.3.3. Direct bilirubin

Principle

Direct bilirubin (conjugated) reacts in acid environment with diazotized sulphalinic acid. The formed coloured azobilirubin is measured photometrically at 546 nm.

Protocol

In test tubes the following were performed:

	Sample	Blank
Direct bilirubin reagent	1000 µL	1000 µL
Nitrite reagent	20 µL	-
Sample	50 µL	50 µL

The reaction mixture was mixed well and incubated in the dark at room temperature for 5 minutes. The absorbance of the sample was measured against respective sample blank within 8 minutes at 546 nm. The concentration wascalculated by subtracting the blank absorbance from the sample absorbance and multiplied by the factor.

3.11.4.3.4. Urea

Measurement was based on the colorimetric end point test method.

Principle

The Berthelot reaction has long been used for the measurement of urea and ammonia. The present method is a modified Berthelot method. The urea colorimetric procedure is a modification of the Berthelot reaction. Urea is converted to ammonia by the use of urease. Ammonium ions then react with a mixture of salicylate, sodium nitroprusside and hypochlorite to yield a blue-green chromophore. The intensity of the color formed is proportional to the urea concentration in the sample.

Protocol

In test tubes the following were performed:

	Blank	Standard	Sample
Urea buffer	1000 µL	1000 µL	1000 µL
Urea enzyme reagent	100 µL	100 µL	100 µL
Standard	-	10 µL	-
Sample	-	-	10 µL
Mixed well and incubated for 5 minutes at 37°C.			
Urea colour developer	1000 µL	1000 µL	1000 µL
Mixed well and incubated for 5 minutes at 37°C.			

The absorbance of sample and standard was measured against reagent blank at 578 nm. The concentration of the standard was then multiplied by sample absorbance and divided by the standard absorbance.

3.11.4.3.5. Creatinine

Measurement was based on the colorimetric kinetic test method developed by Jaffe reaction.

Principle

Creatinine in alkaline solution reacts with picrate to form a coloured complex which absorbs at 500-520 nm. The amount of complex formed is directly proportional to the creatinine concentration.

Protocol

In test tubes the following were performed:

	Blank	Standard	Sample
Sample	-	-	100 µL
Standard	-	100 µL	-
Picrate and buffer reagents	1000 µL	1000 µL	1000 µL

The reaction mixture was mixed well and after 30 seconds at room temperature the initial absorbance was read and read again after 1 minute. The change in absorbance in a minute of the standard and sample were measured against the reagent blank at 500 nm. The concentration of the standard was then multiplied by the product of sample absorbance/standard absorbance.

3.11.4.3. 6. Cholesterol

Measurement was based on the enzymatic colorimetric test method.

Principle

Cholesterol esters are hydrolysed to produce cholesterol. Hydrogen peroxide is then produced from oxidation of cholesterol by cholesterol oxidase. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantypyrine in the presence of phenol and peroxide. The absorption of the red quinoneimine dye is proportional to the concentration of cholesterol in the sample.

Protocol

In test tubes the following was performed:

	Blank	Standard	Sample
Sample	-	-	10 µL
Standard	-	10 µL	-
Enzyme reagent	1000 µL	1000 µL	1000 µL

The reaction mixture was mixed well and incubated for 5 minutes at 37°C. The absorbance of sample and standard was measured against reagent blank at 505 nm. The concentration of the standard was then multiplied by the product of sample absorbance and divided by the standard absorbance.

3.11.4.3.7. High density lipoprotein (HDL)

Measurement was based on the CHOD-PAP tests method

Principle

Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated by the precipitating reagent. After centrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction remains in the supernatant in this phase and is determined by an enzymatic (CHOD-PAP) method.

Protocol

Precipitation step

Test tubes were contained 500 µL of each sample then added 500 µL precipitating reagent was mixed and allows standing for 5 minutes. Centrifuge for 10 minutes at 3000 rpm and determining the cholesterol content by the CHOD-PAP method. Only clear supernatant must be used.

Assay step: in test tubes the following were performed

	Blank	Standard	Sample
Sample	-	-	50 μL
Standard	-	50 μL	-
Enzyme reagent	1000 μL	1000 μL	1000 μL

The reaction mixture was mixed well and incubated for 5 minutes at 37°C. The absorbance of sample and standard was measured against reagent blank at 520 nm. The concentration of the standard was then multiplied by the product of sample absorbance and divided by the standard absorbance and a serum dilution factor.

3.11.4.3. 8. Triglycerides

Measurement was based on the enzymatic colorimetric test method

Principle

Triglycerides are determined after enzymatic hydrolysis with lipases. The quinoneimine indicator if formed from hydrogen peroxide, 4-aminophenazone and 4-chorophenol under the catalytic influence of peroxidase.

Protocol

To test tubes the following was performed:

	Blank	Standard	Sample
Sample	-	-	10 μL
Standard	-	10 μL	-

Enzyme reagent	1000 µL	1000 µL	1000 µL

The reaction mixture was mixed well and incubated for 5 minutes at 37°C. The absorbance of sample and standard was measured against reagent blank at 505 nm. The concentration of the standard was then multiplied by the product of sample absorbance and divided by the standard absorbance.

3.11.4.4. Bacteriological enumeration in colon contents

Colon content of rats from each group were collected for bacteriological enumeration under high aseptic conditions in sterile bottle to avoid any cross contamination. A suspension of 10% (w/v) was made with buffered peptone water. The content was gently homogenized inside a cabinet and serially diluted prior to plating on different agar plates. Subsequent 10-fold serial dilutions of each sample were plated. Media used for total aerobe, total anaerobe, was reported previously by Stanton *et al*. (2003). *Staphylococcus*, *coliform*, *lactobacillus* and *bifidobacteria* enumerated following Liong and Shah (2006) method. While for salmonella, Brilliant green agar was used. Incubation conditions of media used for enumerations are shown in Table (4). All samples were incubated at 37ºC. Anaerobic condition was created in anaerobic jars using gas-generating kits.

Table 4. Media and incubation conditions used for enumeration of different microbiota communities in colon of rats fed different fermented barley beverages

Bacterial group	Type of media	Incubation
Total aerobes*	Nutrient agar	Aerobic
Total anaerobes**	Brain heart infusion agar	Anaerobic****
Coliform**	Macconky agar	Anaerobic****
Salmonella**	Brilliant green agar	Aerobic
Staphylococcus*	Mannitol salt agar	Aerobic
Lactobacillus*	De Man Rogosa Sharpe agar	Anaerobic****
Bifidobacteria*	De Man Rogosa Sharpe agar+ L- cystiene	Anaerobic****

[a]All samples were incubated at 37 ºC.

* Incubation for one day.

** Incubation for two days.

*** Incubation for three days.

****Anaerobic condition was created in anaerobic jars.

3.12. Statistical analysis

One- way ANOVA and two sample paired test were performed to examine significant differences between normally distributed data of replicated independent analysis. Probability level of less than 0.05 was considered significant ($p<0.05$). All data were analysed using vision 16 MINITAB statistical software for windows (2006).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Chemical composition and energy value of barley

Proximate composition and energy value of non-malted and malted barley varieties is shown in Table 5.

4.1.1. Moisture content

The moisture content of non-malted barley was 6.66, 6.43 and 6.08% for Bukur, Balady and Local 46, respectively. After malting process, it was significantly decreased ($p<0.05$) in all varieties recording value of 4.66, 4.44 and 4.35% for Bukur, Balady and Local 46, respectively (Table 5). This finding is similar to that reported by Marconi *et al.* (2014). While Warle *et al.* (2015) and Khatoon and Prakash (2006) reported that the moisture content after malting increased due to the increasing number of hydrated cells within the seed.

4.1.2. Protein content

The Protein content in non-malted barley was found to be in range of 11.40 - 12.62%. However, after malting it decreased significantly ($P <0.05$) from 9.11 into 6.47% in all samples except sample Local 46 where the recorded protein was lower than in other varieties. This finding is in agreement with the results reported by Makeri *et al.* (2013), Marconi *et al.* (2014), Arif *et al.* (2011) and Megat *et al.* (2011) they reported reduction in protein levels of barley by malting. Other studies by Pawar and Machewad (2006) explained that the cause of decreasing protein content was due to leaching process or during transport protein from seeds to roots and then to shoots of plant. On the other hand, many studies conducted by Ghavidel and Prakash (2007) and Kaushik *et al.* (2010) found that, there was increase in protein content during germination process specifically in legume.

Although, Senhofa (2016) observed increase in protein content during malting of several cereals such as wheat, oat, barley and rye.In contract, Jones (2005) stated that barley proteins degraded into amino acids and small peptides as a result of proteolysis enzymes thus protein content decreased in suppar finnding table 5. Further Afify *et al.* (2012) also observed significant decreases in crude protein content by germination of sorghum.

4.1.3. Fat content

Fat content of barley varieties was significantly decreased ($p<0.05$) after malting. The results showed that non-malted barley were 1.94, 1.75 and 1.60% for Bukur, Balady and Local 46, respectively. while the results of malted barley were significant declined so the results were 1.64, 1.68 and 1. 49 for Bukur, Balady and Local46 respectively (Table 5).These results were similar to those reported by Arif *et al.* (2011) and Warle *et al.* (2015) they found that fat content decreased after malting of barley varieties . Also, Youssef *et al.* (2012) indicated that the changes in lipid fractions might be due to hydrolysis of triglycerides and polar lipid components into simpler compounds during germination process.

4.1.4. Ash content

After malting process, ash content was decreased significantly ($p<0.05$) in all barley varieties except Balady (Table 5). The ash content of non-malted barley were 2.48, 2.12 and 2.52% for Bukur, Balady and Local46, respectively. Although, many studies by Arif*et al.* (2011) and Megat *et al*. (2011) reported that ash content decreased due to soaking process.Other study by Pawar and Machewad (2006) showed that the cause of decreasing the ash content of barley by germination grains was the solubility of minerals in water and leaching out during processing. Whereas, Tatsadjieu *et al*. (2004) found decrease in ash content by germination

loss of the minerals due to rootlet and washing of the barley by water undertaken to reduce the sour smell during the germination period.

4.1.5. Fiber content

The fiber content of malted barley was increased significantly ($p<0.05$) as compared with non-malted samples (Table 5). The results were supported by the findings of Arif *et al.* (2011) who studied the effect of malting on the nutrient profile of barley, and observed significant increase from 5.90 to 8.15% in the crude fiber content. Also, a slight increase in total dietary fibre content has been previously observed by Hubner *et al.* (2010) by germination process. They attributed that to the loss of starch and synthesis of fibrous compounds for the development of rootlets in growing plants. Rootlets are usually removed for the preparation of barley flour. However, Azizah and Zainon (1997) reported that dietary fiber was decreased in soaked wheat, and barley but conversely increased in soaked rice and soy bean. Therefore, Grove *et al.* (2003), Moore and Jung (2001) reported that variation in the content of the fiber is due to different in barley hull between varieties in addition different agronomic practices.

4.1.6. Carbohydrate content

The results in table 5 explained that after malting process carbohydrate levels in all barley varieties were significantly ($p<0.05$) increase compare non-malted ones. Although, decrase in starch was reported by Makeri *et al.* (2013) due to germination, the decrease in moisture and other components such as protein, ash and fat are the main factors that impact on increased carbohydrate content as percentage in all malted barley variety.

4.1.7. Energy value

The energy value of different malted and non-malted barley varieties is presented in Table (5).The calculated energy ranged from 350.86 kcal/100g in Bukur to 353.02kcal/100g in Local 46 non malted varieties. After malting, it was increase significaltly ($P < 0.05$) in all varieties. The amount to be taken daily to meet the daily energy requirements for adult men (3200 kcal) and for adult women (2300 kcal)) as recommended by FAO/WHO(2003).

4.2. Reducing, non-reducing and total sugars contents of malted barley

Table 6.shows total sugars, reducing and non-reducing sugar contents of malted barley. Reducing sugar for non-malted barley content was (0.67, 0.25 and 0.37 mg/100g for Bukur, Balady and Local 46 respectively. while for non-reducing was 0.41, 0.48 and 0.32 mg/100g for Bukur, Balady and Local46, respectively. Malting process caused significant ($P < 0.05$) increases in total sugars and non-reducing sugars, while the related in reducing sugar was decreased by malting (Table 6). These changes in sugars content may be due to mobilization and hydrolysis of polysaccharides during soaking and germination processes (Hooda and Jood, 2003). Also, Nikita and Punia (2006) revealed that the reducing sugars, non-reducing sugars, and total sugars content of non-malted and malted wheat varied from 0.52 to 0.58, 3.87 to 4.45 and 4.43 to 5.06 respectively.

4.3. Minerals content

Table (7) showed that minerals content such as Ca, K, Na, Cu, Fe, Mg, Mn and Zn (mg/100g) of malted barley. Potassium, recorded the highest value among other minerals. On the other hand, Copper was recorded lowest value. However the results showed lower levels of minerals in malted than in non- malted ones. These results are similar to that reported by Ereifej and Haddad (2000) in study made in Jordon and Morocco. It can be observed that minerals content decreased by

germination which may be due to their destruction in water (Urbano *et al.* 2005). Study by Jood and Kalra (2001) revealed that iron content was poor in barley whereas calcium was present in good amount.

4.4. Microbiological safety of non malted and malted barley

The results of microbiological safety of malted barley are shown in Table 8.In this study, the total bacterial viable count of non-malted barley was 4.02, 4.22 and 4.70 cfu/g for Bukur, Balady and Local 46, respectively. While total count of bacteria in malted barley was 6.064, 6.55 and 6.15 cfu/g forBukur, Balady and Local46, respectively. During malting process, the total count of bacteria increased significantly ($p<0.05$) by malting Table 8. Yeasts and moulds, Total coliform and *E.coli* count showed a significant different ($P<0.05$) by malted barley compeared to non-malted one. Victor *et al.* (2013) and Batool *et al.* (2012) found similar results of total count bacteria where of maize and wheat flour increasing by malting. *Salmonella* spp. were not detected in both non-malted and malted barley varieties. However, similar result was reported for *salmonella* absent in malted wheat flour by Aydin *et al.,* (2009).

Table 5. Chemical composition and energy value of different non malted and malted barley

Component (%)	Barley	Varieties		
		Bukur	**Balady**	**Local 46**
Moisture	Non- malted	$6.66\pm.16^{a}_{a}$	$6.43\pm.15^{b}_{a}$	$6.0878\pm.50^{c}_{a}$
	Malted	$4.66\pm.016^{a}_{b}$	$4.44\pm0.04^{b}_{b}$	$4.35\pm0.021^{c}_{b}$
Protein	Non- malted	$12.62\pm.16^{a}_{a}$	$11.40\pm.20^{b}_{a}$	$11.53\pm0.21^{b}_{a}$
	Malted	$9.11\pm0.11^{a}_{a}$	$7.83\pm.090^{b}_{b}$	$6.47\pm.13^{c}_{b}$
Fat	Non- malted	$1.94\pm0.09^{a}_{a}$	$1.75\pm0.073^{a}_{a}$	$1.60\pm0.\ 22^{a}_{b}$
	Malted	$1.64\pm0.15^{a}_{b}$	$1.68\pm0.025^{a}_{b}$	$1.49\pm0.11^{b}_{a}$
Ash	Non- malted	$2.72\pm.02^{a}_{b}$	$2.52\pm0.025^{b}_{a}$	$2.45\pm0.02^{b}_{b}$
	Malted	$2.48\pm0.015^{a}_{a}$	$2.47\pm0.03^{b}_{a}$	$2.12\pm0.015^{c}_{a}$
Fiber	Non- malted	$3.13\pm0.04^{a}_{a}$	$2.80\pm0.29^{b}_{a}$	$3.52\pm0.02^{c}_{a}$
	Malted	$4.02\pm0.015^{a}_{b}$	$4.24\pm0.02^{b}_{b}$	$4.47\pm0.03^{c}_{b}$
Carbohydrate	Non- malted	$73.15\pm0.13^{a}_{a}$	$75\pm0.390^{b}_{a}$	$75.22\pm0.726^{b}_{a}$
	Malted	$75.63\pm0.12^{a}_{b}$	$79.25\pm0.25^{b}_{b}$	$80.92\pm0.27^{c}_{b}$
***Energy(Kcal/100g)**	Non -malted	$350.86\pm0.67^{a}_{a}$	$352.6\pm0.99^{a}_{a}$	$353.02\pm2.09^{a}_{a}$
	Malted	$364.97\pm0.86^{a}_{b}$	$363.83\pm0.15^{a}_{b}$	$362.90\pm1.07^{b}_{b}$

Values are mean ± SD for triplicates independent runs.

*Means carrying the same superscript letter in row for each specific component are not signifacintly different ($p < 0.05$).

*Means carrying the same subscript letter in column for the same variety are not signifacintly different ($p < 0.05$).

* The energy value was calculated using factors of 4.00 kcal/g for protein, 9.00 kcal/g for fat and 4.00 kcal/g for total carbohydrates.

Table 6. Reducing, non-reducing and total sugars (mg/100g) in non malted and malted barley

Component	Barley	Varieties		
		Bukur	Balady	Local 46
Reducing Sugar	Non-malted	$0.67 \pm 0.08^{a}_{a}$	$0.48 \pm 0.00^{b}_{b}$	$0.37 \pm 0.00^{c}_{a}$
	Malted	$0.42 \pm 0.01^{a}_{b}$	$0.25 \pm 0.009^{b}_{a}$	$0.32 \pm 0.050^{c}_{b}$
Non- Reducing sugar	Non-malted	$0.63 \pm 0.13^{a}_{a}$	$0.85 \pm 0.057^{b}_{a}$	$0.98 \pm 0.00^{c}_{a}$
	Malted	$2.17 \pm 0.17^{a}_{b}$	$2.74 \pm 0.05^{b}_{b}$	$1.72 \pm 0.13^{c}_{b}$
Total sugar	Non-malted	$1.30 \pm 0.06^{a}_{a}$	$1.10 \pm 0.00^{b}_{a}$	$1.35 \pm 0.02^{c}_{a}$
	Malted	$2.59 \pm 0.00^{a}_{b}$	$3.23 \pm 0.057^{b}_{b}$	$2.04 \pm 0.00^{c}_{b}$

Values are mean ± SD for triplicates independent runs.

*Means carrying the same superscript letter in row for each specific component are not signifacintly different ($p < 0.05$).

*Means carrying the same subscript letter in column for the same variety are not significantly different ($p < 0.05$).

Table 7. Mineral content (mg/100g) of non-malted and malted barley

Minerals	Barley	Varieties		
		Bukur	Balady	Local 46
Ca	Non-malted	$27.17\pm1.37_{a}^{a}$	$21.11\pm4.88^{b}_{a}$	$24.35\pm1.38^{a}_{a}$
	Malted	$26.66\pm 0.54_{a}^{a}$	$20.95\pm5.88^{b}_{a}$	$22.52\pm0.20^{a}_{b}$
K	Non-malted	$298.67\pm0.81^{a}_{a}$	$266.68\pm1.17^{b}_{a}$	$252.67\pm4.02^{c}_{a}$
	Malted	$277.27\pm5.54^{a}_{b}$	$249.82\pm9.86^{b}_{b}$	$220.11\pm0.60_{b}$
P	Non-malted	$228\pm5.75^{a}_{a}$	$231.6\pm1.10^{a}_{a}$	$209.81\pm0.95^{b}_{a}$
	Malted	$210.99\pm1.21^{a}_{b}$	$228.15\pm5.07^{b}_{a}$	$213.66\pm2.31^{a}_{a}$
Na	Non-malted	$9.60\pm0.63^{a}_{a}$	$6.56\pm0.26^{b}_{a}$	$8.19\pm0.63^{c}_{a}$
	Malted	$6.56\pm0.25^{a}_{b}$	$5.38\pm0.095^{b}_{b}$	$6.56\pm0.26^{a}_{b}$
Cu	Non-malted	$0.95\pm 0.29^{a}_{a}$	$0.54\pm0.03^{b}_{a}$	$0.68\pm0.02^{c}_{a}$
	Malted	$0.73\pm0.05^{a}_{b}$	$0.49\pm0.011^{b}_{a}$	$0.51\pm0.1^{b}_{b}$
Fe	Non-malted	$2.54\pm 0.020^{a}_{a}$	$1.93\pm0.68^{b}_{a}$	$2.12\pm0.01^{b}_{a}$
	Malted	$1.96\pm0.15^{a}_{b}$	$1.55\pm0.12^{a}_{b}$	$2.04\pm0.03^{a}_{b}$
Mg	Non-malted	$64.27\pm2.31^{a}_{a}$	$52.64\pm0.39^{b}_{a}$	$68.53\pm0.92^{c}_{a}$
	Malted	$60.53\pm0.68^{a}_{a}$	$51.37\pm0.95^{b}_{a}$	$60.87\pm0.77^{a}_{b}$
Mn	Non-malted	$1.29\pm 0.02^{a}_{a}$	$1.15\pm 0.05^{b}_{a}$	$1.23\pm0.06^{a}_{a}$
	Malted	$1.08\pm0.01^{a}_{b}$	$1.02\pm0.051^{b}_{b}$	$1.02\pm0.01^{b}_{b}$
Zn	Non-malted	$1.65\pm 0.13^{a}_{a}$	$1.49\pm0.12^{a}_{a}$	$1.77\pm0.26^{a}_{a}$
	Malted	$1.48\pm0.30^{a}_{a}$	$1.55\pm0.18^{a}_{a}$	$1.31\pm0.1^{a}_{b}$

Values are mean ± SD for triplicates independent runs.

*Means carrying the same superscript letter in row for each specific component are not signifacintly different ($p < 0.05$).

*Means carrying the same subscript letter in column for the same variety are not signifacintly different ($p < 0.05$).

Table 8. Microbiological safety of non-malted and malted barley

Microbiological test	Barley	Varieties		
		Bukur	Balady	Local 46
Total count (cfu/g)	Non-malted	$4.02 \pm 0.02^{a}_{a}$	$4.22 \pm 0.16^{a}_{a}$	$4.7 \pm 0.03^{b}_{a}$
	Malted	$6.06 \pm 0.04^{a}_{b}$	$6.56 \pm 0.29^{b}_{b}$	$6.15 \pm 0.08^{a}_{b}$
Yeast and moulds (cfu/g)	Non-malted	$3.95 \pm 0.11^{a}_{a}$	$4.02 \pm 0.49^{a}_{a}$	$3.55 \pm 0.13^{a}_{a}$
	Malted	$4.07 \pm 0.008^{a}_{b}$	$4.19 \pm 0.009^{b}_{b}$	$4.10 \pm 0.08^{a}_{b}$
Coliform (MPN/g)	Non-malted	$3.35 \pm 0.77^{a}_{a}$	$110 \pm 0^{b}_{a}$	$6.1 \pm 3.2^{c}_{a}$
	Malted	$<110 \pm 0.00^{a}_{b}$	$<110 \pm 0.00^{a}_{b}$	$<110 \pm 0.00^{a}_{b}$
E.coli **(MPN/g)**	Non-malted	$5.6 \pm 3.5^{a}_{a}$	$4.1 \pm 0.40^{a}_{a}$	$2.6 \pm 0.24^{a}_{a}$
	Malted	$2.05 \pm 0.05^{a}_{a}$	$4.4 \pm 0.73^{b}_{a}$	$110 \pm 00^{c}_{b}$
Salmonella	Non-malted	N.D	N.D	N.D
	Malted	N.D	N.D	N.D

Values are mean ± SD for triplicates independent runs.

[*]Means carrying the same superscript letter in each row for specific microbiological test are not signifacintly different ($p < 0.05$).

*Means carrying the same subscript letter in each column for the same variety are not signifacintly different ($p < 0.05$).

N.D = Not detected.

4.5. Phytochemicalsal content

Table (9) presents the tannin, phytate, polyphenols and flavenol content in non – malted and malted barley varieties. The results showed significant ($p<0.05$) decreases in all Phytochemicals all levels after malting. These results are supported by finding by Idris *et al.* (2005) on effect of germination on tannin content of sorghum cultivars and observed reduction up to 61% and 34% in two different varieties.Ogbonna *et al.* (2012) revealed a decrease in tannin content due to leaching loss during steeping. According to Adeyemo and Onilude (2013), most cereals and legumes contain some appreciable amounts of phytate, tannins, trypsin inhibitors and other anti-nutrients which may be effectively reduced by germinatation, thereby improving the nutritional quality of these cereals and legumes. Utilization of phytate as source of inorganic phosphate for germination, phytase activity, and leaching loss during soaking may result in reduction in phytate (Bau *et al.,* 1997). Phytochemicals can negatively affect bioavailability of nutrients (Liang *et al.*, 2008). On the other hand, prolonged soaking can help reduce the content of these phytochemicals through leaching (Ogbonna *et al.,* 2012). The reduction in the tannin and phytic acid content in malted cereals, make minerals are made more bioavailable, thereby increasing the nutritional value of the food (Ogbonna *et al.,* 2012; Oghbaei and Prakash, 2016). Lestienne *et al.* (2005) investigated the effect of soaking wholegrains (sorghum, millet, barley, rice, and maize) on phytate content in order to evaluate the effectiveness of this treatment for improving the bioavailability of micro-nutrients such as iron and zinc. They recorded a significant reduction ($P < 0.05$) in phytate content (between 17% and 28%) after soaking whole seeds for 24 h at 30°C. also, Elmaki and others (1999) reported that soaking of sorghum seeds in water and germination were found to be effective in reducing tannin content of high-tannin

cultivars and caused an appreciable improvement in the in vitro protein digestibility.

4.6. Growth of *Bifidobacterium longum* BB536 during fermentation of different barley beverages

The results in table 10. shows that there was significant increase ($p<0.05$) in *Bifidobacterium longum* BB536 viable count by extending fermentation of different barley beverages. The maximum growth of *B. longum* BB536 was attained at 18h in all types of fermented beverages, the maximum growth was obtained of Re-constituted skim milk and Bukur, Balady and Local 46 barley beverages was 8.13, 8.19, 8.08 and 8.17 log CFU/ml, respectively. The rate of *B. longum* BB536 increased in different fermented beverages was 2.40, 2.50, 3.06 and 2.38% in fermented Re-constituted skim milk, Bukur, Balady and Local 46 barley beverages, respectively. the high growth of B. *longum* BB536 was in Bukur variety because it contains the highest total sugars and non-reducing sugar as shown in Table (6). Barley beverage also contain saccharides specifically monosaccharides and disaccharides (Charalampopoulos *et al.*2002; Rathore *et al.* 2012). After that 18h fermentation, the strain level declined in all types of fermented barley beverages. However, the reduction in growth after 18h fermentation is mainly referred to the accumulation of acids or reduction of availability of nutrient required for the growth as stated by Kabeir *et al.* (2005). Similar maximam growth for other *Bifidobacterium* strains in peanut milk and skim milk supplemented with fructooligosaccharides had amounted to about 8.39 cfu/ml after 24h incubation as reported by Kabeir *et al.*(2014). Also, Wang *et al.*(2002) and Laine *et al.*(2003) reported that the growth of *Bifidobacterium* strains in soymilk and oat based medium have amounted to about 7 log cfu/ml after 24h incubation. Other study by Arora *et al.*(2010) revealed that a number of 7.58×10^{8} cfu/ml of *Lacidophilus* in fermented food mixture formulated with

germinated barley flour was observed. Also, Patel *et al.* (2004) reported a higher maximum growth of *L. plantarum* in malt, barley and wheat of 9.15, 8.46 and 8.39 log cfu/ml, respectively. Kedia *et al.* (2008) reported a maximum growth of *B. lactis* of 9.16 log10 cfu/ml in white oat beverage. Rice-based medium were also supported the growth of *L. plantarum NCIMB 8826* with a biomass value of approx 10.4 log10cfu/ ml (Saman *et al.,* 2011). Further, Rathore *et al,* (2012) reported *L. plantarum* population of 8.59 log cfu/mL when malt flour beverage was fermented with this strain for 24 h. The international standard FIL/IDF describe that the probiotic products should contains minimum of 10^6 viable probiotic bacteria per gram of product at the time of consumption for health and functional claiming (Samona and Robinson, 1991; Roy, 2005). The viable cell levels in the final fermented beverages (10^8-10^9 cfu/ ml) were above the minimum dose (10^6 cfu/ml) to maintain the intestinal population and to ensure that the consumer will derive health benefits (Ukwuru and Ohaegbu, 2018). Thus, for all barley varieties beverages fermented with *B.longum* BB536 for 18h fulfill the probiotic number recommended to claim health beneficial.

Table 9. Phytochemicals (mg/100g) content in non-malted and malted barley varieties

parameter	Treatment	Varieties		
		Bukur	**Balady**	**Local 46**
Tannin	Non-Malted	$219\pm 4.02^{a}_{a}$	$233.12\pm2.93^{a}_{a}$	$212.34\pm2.00^{a}_{a}$
	Malted	$90.94\pm0.82^{a}_{b}$	$68.15\pm2.42^{b}_{b}$	$89.673\pm0.56^{c}_{b}$
Phytate	Non-Malted	$198.59\pm2.03^{a}_{a}$	$210.56\pm0.12^{b}_{a}$	$216.16\pm4.95^{c}_{a}$
	Malted	$96.25\pm4.87^{a}_{b}$	$89.38\pm0.00^{c}_{b}$	$93.06\pm4.55^{b}_{b}$
Polyphnol	Non-Malted	$167.34\pm0.96^{a}_{a}$	$159.05\pm2.47^{a}_{a}$	$175.58\pm4.12^{a}_{a}$
	Malted	$57.67\pm4.74^{a}_{b}$	$40.14\pm0.66^{c}_{b}$	$51.68\pm0.54^{b}_{b}$
Flavenol	Non-Malted	$96.64\pm1.72^{a}_{a}$	$67.21\pm2.21^{c}_{a}$	$84.33\pm2.65^{b}_{a}$
	Malted	$30.22\pm 0.95^{b}_{b}$	$30.42\pm1.04^{a}_{b}$	$22.5\pm1.84^{c}_{b}$

Values are mean ± SD for triplicates independent runs.

*Means carrying the same superscription letter in each row for each specific parameter are not signifacint different ($p < 0.05$).

*Means carrying the same subscription letter in each column for the same variety are not signifacint different ($p < 0.05$).

Table 10. The growth of *Bifidobacterium longum* BB536 (log CFU/ml) during fermentation of different barley beverages

Time(h)	Fermented Beverages			
	re-constituted skim milk	Barley varieties		
		Bukur	Balady	Local 46
0	5.73±0.03[e]	5.71±0.49[d]	5.02±0.005[e]	5.79±0.02[e]
6	7.35±0.56[c]	7.18±0.05[b]	7.28±0.03[c]	7.29±0.01[d]
12	7.98±0.19[b]	8.19±0.02[a]	7.94±0.07[b]	8.14±0.04[b]
18	8.13±0.01[a]	8.21±0.04[a]	8.08±0.05[a]	8.17±0.06[a]
24	6.17±0.05[d]	7.09±0.02[c]	7.11±.07[d]	7.67±0.54[c]

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

4.7. Physiochemical properties

4.7.1. Changes in pH levels during fermentation of different barley beverages

During fermentation process with strain *Bifidobacterium longum* BB536 there were significant (P<0.05) decreases in pH levels of all types of beverages by extended fermentation period to 24 h (Table 11). The initial pH was 6.55, 5.52, 5.57 and 5.72 pH for fermented Re-constituted skim milk, Bukur, Balady and Local 46 barley beverages respectively. The pH decrease at maximum growth (18h) of *Bifidobacterium longum* BB536 to 4.10, 3.57, 3.55 and 3.60 in fermented re-constituted skim milk, Bukur, Balady and Local 46 barley beverages, respectively.The decreases in pH are due to increased acids production during fermentation process as a result of fermenting sugar by *Bifidobacterium longum* BB536, and production of acetic and lactic acids. The pH of malted-roasted beverages was generally higher than that obtained for malt beverages from barley and oats (pH 4.01). Study by Obuzor and Ajaezi (2010) obtained for some commercial carbonated non-alcoholic malt beverages recorded pH range of 4.4 – 4.6. These results are in consistent with recorded by Rozada-Sanchez *et al.,* (2008) on *Bifidobacterium spp*. They studied production of a potentially probiotic malt-based beverage fermented for 14 hours where pH ranges between 4.30 and 4.10. Also, Buruleanu (2009) reported that pH value of the carrot juices was decreased from an initial value of 6.45 to 4.3 after 48 hours fermentation with *Bifidobacterium sp.* Angelov *et al.*(2005) revalued pH 4 and 4.5, due to fermentation with *Bifidobacterium* spp. for a period of 12–14 hours.

4.7.2. Total soluble solids (TSS) levels during fermentation of different barley beverages

Table 12. shows the changes in TSS during fermentation of different formulated beverages with *B. longum* BB536.There were significant (P<0.05) decrease in

TSS levels in all types of fermented beverages by extended fermentation period to 24 h. The rate of TSS decrease at maximum growth were 2, 0.3, 1.07 and 1.47 % in fermented re-constituted skim milk, Bukur, Balady and Local 46 barley beverages respectively.The reduction in TSS could be due to enzymatic activity of the strain during fermentation process (Kabeir *et al*., 2005). A similar decrease in TSS during traditional microbial processing of Malwa beverage by fermentation was detected(Muyanja *et al.,* 2010).

4.7.3. Titratable acidity during fermentation of different barley beverage with *Bifidobacterium longum* BB536

Table 13.shows the titratable acidity of different fermented beverages. There were significant ($p<0.05$) increases in titrtable acidity by extended fermented period to 24h. At maximum growth of strain BB536 (18h), the rates of titratable acidity increase were 0.27, 0.23, 0.14 and 0.17% in fermented re-constituted skim milk, Bukur, Balady and Local 46 barley beverages, respectively. The increase in titrable acidity content in fermentation might be due to the production of organic acids by *Bifidobacterium longum* (Sefa Dedeh *et al.,* 2003). Study by Farnworth *et al.* (2007) found that after 12 hours fermentation with *bifidobacteria* of soy beverages a titratable acidity of 0.38 – 0.39% was recorded. The increase in acidity causes decrease in the number of *Bifidobacterium spp*. growth when a pH of 4.0–3.6 is reached. *Bifidobacterium* is less acid tolerant and its growth is retarded at low pH of 5.0–4.5 (Shah, 2000).

Table 11. Changes in pH levels during fermentation of different barley beverages

Time (h)	Fermented beverages			
	re-constituted skim milk	Barley varieties		
		Bukur	Balady	Local46
0	6.55±0.005^{a}	5.52±0.015^{a}	5.57±0.005^{a}	5.72±0.005^{a}
6	6.30±0.005^{b}	4.60±0.005^{b}	4.61±0.005^{b}	5.12±0.005^{b}
12	5.81±0.005^{c}	4.21±0.010^{c}	4.17±0.011^{c}	4.81±0.005^{c}
18	4.18±0.005^{d}	3.57±0.005^{d}	3.55±0.010^{d}	3.60±0.005^{d}
24	4.10±0.005^{e}	3.36±0.005^{e}	3.41±0.005^{e}	3.47±0.005^{d}

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

4.8.Total sugar during fermentation of different beverages with *Bifidobacterium longum* BB536

The result in Table 14 shows significant (P<0.05) decrease in total sugar levels of all fermented beverages by fermentation to maximum strain BB536 growth. The rates of sugar decreased ascompared to initial growth were 0.02, 0.02, 0.04, and0.02 in fermented re-constituted skim milk and Bukur, Balady and Local 46 fermented beverages, respectively. The strain BB536 fermented sugar and produces organic acids, mainly acetic, lactic, propunic, butyric and other organic acids (Sefa-Dedeh *et al.,* 2003). However, these variations in total sugar reduction refer to strain activity. Some strains break down complex polysaccharide during the fermentation that correlated well with the decrease in TSS.

4.9. Proximate composition of different barley beverages fermented with *Bifidobacterium longum* BB536

Table 15 shows the proximate composition of different barley beverages in re-constituted skim milk fermented with *B. longum* BB536 at initial (0h) and maximum growth time (18h). The result presented in table 15, revealed significant (p>0.05) changes in some component chemical content of fermented beverages. The result revealed significant (p<0.05) increases in moisture and protein content of all fermented beverages at maximum growth as compared to initial. This increase in moisture might indicate high enzymatic activity that break down the macro-component into simple and release of water (Ibraheem *et al.,* 2015). Also, the increase of in all fermented beverages is due to microbial growth types.While fat, ash, fiber and carbohydrate decreased by fermentation in fermented beverage (Table 15).

Table 12. Total soluble solids (%) levels during fermentation of different barley beverages

Time (h)	Fermented beverages			
	Re-constituted skim milk	Barley Varieties		
		Bukur	Balady	Local 46
0	7.9±0.10[a]	4.3±0.10[a]	4.06±0.05[d]	5.5±0.10[b]
6	6.06±0.11[b]	3.9±0.100[c]	5.46±0.05[b]	6.1±0.10[a]
12	5.9±0.10[c]	4.06±0.05[b]	6.13±0.05[a]	4.03±0.05[d]
18	5.9±0.10[c]	4.00±0.10[b]	5.13±0.05[c]	4.03±0.20[d]
24	5.56±0.20[d]	3.43±0.11[d]	5.16±0.15[c]	4.06±0.05[c]

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

Table 13. Titratable acidity (%) levels during fermentation of different barley beverages

Time (h)	Fermented beverages			
	Re-constituted skim milk	Barley varieties		
		Bukur	Balady	Local 46
0	$0.13{\pm}0.05^{e}$	$0.10{\pm}0.00^{e}$	$0.16{\pm}0.06^{d}$	$0.20{\pm}0.1^{e}$
6	$0.20{\pm}0.00a^{d}$	$0.13{\pm}0.0577^{d}$	$0.23{\pm}0.06^{c}$	$0.23{\pm}0.05^{d}$
12	$0.27{\pm}0.057^{c}$	$0.23.0.050^{c}$	$0.30{\pm}0.00^{b}$	$0.30{\pm}0.05^{c}$
18	$0.40{\pm}0.00^{b}$	$0.33{\pm}0.057^{a}$	$0.30{\pm}0.00^{b}$	$0.37{\pm}0.05^{b}$
24	$0.60{\pm}0.00^{a}$	$0.30{\pm}0.00^{b}$	$0.43{\pm}0.06^{a}$	$0.43{\pm}0.06^{a}$

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren’t significantly different at $p<0.05$.

Table 14. Total sugars (mg/100g) during growth of *Bifidobacterium longum BB536* in different beverages

Time(h)	Fermented barley beverages			
	Re-constituted skim milk	Bukur	Balady	Local 46
Initial	0.17±0.004[a]	0.23±0.005[a]	0.25±0.010[a]	0.21±0.006[a]
Maximum growth (18h)	0.15±0.004[b]	0.20 ±0.004[b]	0.21± 0.005[b]	0.19 ±0.00[b]

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

Table 15. Proximate composition of different barley beverages fermented with *Bifidobacterium longum* BB536 growth

Component (%)	Types of beverages							
	Re-constituted Skim milk		Bukur		Balady		Local 46	
	Initial	Maximum	Initial	Maximum	Initial	Maximum	Initial	Maximum
Moisture	86.20±0.02[a]	86.22±0.10[a]	86.22±0. 05[a]	86.28±0.01[b]	86.08±0.01[a]	86.14±0.01[b]	86.21±0.05[a]	86.45±0.29[b]
Protein	3.35±0.1[a]	3.48±0.01[b]	3.15±0.00[a]	3.19±0.025[b]	3.18±0.005[a]	3.23±0.02[b]	3.11±0.015[a]	3.20±0.02[b]
Fat	0.27±0.00[a]	0.22±0.07[a]	0.95±0.015[a]	0.89±0.00[b]	0.89±0.00[b]	0.62±1.13[a]	0.89±0.00[a]	0.88±0.02[a]
Ash	0.86±0.05[a]	0.79±0.02[a]	0.24±0.01[a]	0.22±0.01[b]	0.26±0.02[a]	0.24±0.00[a]	0.21±0.01[a]	0.19±0.005[a]
Fiber	0.00±0.00[a]	0.00±0.00[a]	0.17±0.00[a]	0.13±0.015[b]	0.19±0.00[a]	0.16±0.005[b]	0.16±0.01[a]	0.12±0.005[b]
CHO	9.36±0.07[b]	9.22±0.05[a]	9.31±0.04[b]	9.24±0.02[a]	9.62±.14[a]	9.37±0.01[b]	9.36±0.10[a]	9.22±0.28[a]

Values are mean ± SD for replicate independent runs.

Values that carry different superscript letters in the same row for each specific type of beverages are significantly different at $p<0.05$.

4.10. Survival of *Bifidobacterium longum* BB536 (log cfu/ ml) during refrigeration storage of different fermented beverages

Table 16 shows the viable counts of *B. longum* BB536 for over 21 days during refrigeration storage of different fermented barley beverages to assess the shelf life. There was significant reduction in viable counts of fermented beverages during the storage as compared to the initial level at the begnning. The viable counts of *bifidobacterium longum BB536* in the first week of the refrigeration storage were 7.79, 7.89, 7.37, 7.40 cfu/ml in Re-constituted skim milk and Bukur, Balady, and Local46 fermented barley beverages, respectively. It is clear that the rates of reduction were differed among different fermented beverages. Moreover *Bifidobacterium longum* BB536 reductions recorded in the second week of the refrigerated storage recorded value of 0.23, 0.82, 0.74 and 0.41 log cfu/ml of Re-constituted skim milk and Bukur, Balady, and Local 46 fermented barley beverages, respectively. The final viable count of *B. longum* BB536 in fermented barley beverages (Bukur, Balady and Local 46) after three weeks refrigeration storage were above the minimum number required to in probiotic food to exert health benefits upon consumption which is 10^6 cfu/ml. With respect to dairy products, a therapeutic dose of minimum 10^9 cfu/day or consumption of 100g or ml/day should translate to a food containing at least 10^7 cells per g or ml (Jayamanne and Adams, 2006; Raeisi *et al.,* 2013). Generally, the results presented here support data obtained by others. For example, Kabeir *et al.* (2005) they reported significant growth of a *Bifidobacterium* strain in fermented porridge made of rice flour and skim milk, reaching a count of 9.9 log cfu/mL, and decreasing by 0.9 log cfu/ml during a two-week storage period. However, Akaline *et al*., (2004) noted a significant reduction on *B.longum* BB536 in yogurt after one week refrigeration. Lankaputhra *et al.* (1996) observed that viability of *B. infantis* in 12% skim milk at pH 4.3 decreased by 30% after 12 days of storage

at 4ºC. After 24 days at the same temperature the counts decreased by more than 82%. On other hand, Matta *et al.* (2012) developed a symbiotic beverage based on rice and oats, with the addition of fructooligosaccharides and acacia gum and obtained 10^7 CFU/ 1g total viable count of probiotic microorganisms after 22 days of storage. The main factors for loss of viability of probiotic organisms during storage have been attributed to the decrease in the pH of the medium and accumulation of organic acid produced by fermentation (Yoon *et al.,* 2004). In their results by Elsanhoty *et al.* (2009); Arena *et al.*(2014) attributed the decrease in the viability of *Bifidobacterium lactis* to their sensitivity towards low pH arising mainly from the high concentrate of lactic and acetic acids. The possibility of growth ability may change the chemical composition of the fermentation medium to better survival environment (Saccaro *et al.,* 2011). On the other hand, Mortazavian *et al.* (2007) investigated the effect of cold storage temperature on the viability of probiotics in yoghurt and reported that the highest viability of *L. acidophilus* after 20 days was attained at 2ºC.Whereas for *Bifidobacterium lactis* the highest viability was obtained when yoghurt was stored at 8ºC.The variances in survival were interpreted by the metabolic activity of *Bifidobacterium* in different fermented products, which might be affected by the composition and availability of nitrogen and carbon sources in growth media as stated by Chou and Hou (2000).According to Angelov *et al.* (2005) *L. plantarum* B28 has the ability to grow in fresh oat-based drink with cell counts of 9.97 log cfu /ml and maintain high viability (10^6–10^7 cfu /ml ; pH 4.5) during 21 days of storage. In another study, four strains of bifidobacteria (*B. adolescentis, B. infantis, B. breve and B. longum*) were used to produce probiotic malt-based beverage. All the bifidobacteria strains showed viability of 9 log cfu/ml in the final product which was greatly exceed the recommended value of probiotic products (10^6 –10^7 cfu/ml) (Rozada *et al.,* 2008). However, the products were considered safe

according to the standards and no visual spoilage was observed during storage period in any of the developed probiotic products.

4.11. Changes of pH during storage of different fermented beverages

The reduction of pH during the refrigeration storage of different fermented beverages is presented in Table 17. There was significant ($p<0.05$) reduction in pH of all types of fermented products during the two weeks refrigeration temperature (4ºC). The rates of pH reduction in the first week were 0.09, 0.19, 0.09, , and 0.08 pH in fermented Re-constituted skim milk and Bukur, Balady Local 46 fermented barley beverages, respectively. While the reductions recorded at the end of refrigeration storage were 3.73, 3.15, 3.22, and 3.36 in fermented Re-constituted skim milk, Bukur, Balady and Local 46 fermented barley beverages, respectively. Akalin *et al.* (2004) reported that probiotic microorganisms reduced the pH of yogurt from 4.51-4.40, after 28 days refrigerated storage at 4°C. Some factors can modify the pH value of fermented products, such as starter cultures, addition of different substrate ratios, processing conditions and storage temperature (Chauhan *et al.,* 2007). The reduction of pH is mainly due to the fermentation of sugars and accumulation of acid. In fermented beverages, pH is relevant for the microbiological stability, anti-food-borne pathogens and acid-sensitive microorganisms, and may be directly correlated with the products taste (Salmerón *et al.* 2014; Farnworth *et al.* 2007). Lankaputhra *et al.* (1996) reported survival of three out of nine bifidobacteria strains in the pH range of 4.3–3.7. That is why *Bifidobacterium* maintain a relatively acid pH in large intestine, thus preventing the proliferation of pathogens. It produces lactic acid, acetic acid, hydrogen peroxide, and bactericides (Bullen *et al.*, 1976). The reduction in pH is mainly due to the

relatively acidic pH in the large intestine, thus preventing the growth ofpathogens.

4.12. Changes in total soluble solids (%) during refrigeration storage of different fermented beverages

The results in Table 18. present the total sugars during the refrigerated storage period of different fermented beverage. There was significant ($p<0.05$). Decrease in the total sugars of different fermented beverages which correlated well with reduction of TSS in Table (18). The percentages of sugar decrease in the first week were 0.90, 1.06, 0.80 and 1.78 in Re-constituted skim milk and Bukur, Balady, Local 46 fermented beverages respectively. The percentages of sugar reduction in the second week were higher as compared to that of the first week recording values of 1.47, 0.91, 0.07, and 0.48 in the fermented Skim milk, Bukur, Balady and Local 46 beverages, respectively. These results could have an important nutritional significance, because theyindicate that the prebiotic addition improves bifidobacterial enzyme activity during the barley beverage fermentation (Donkor *et al.,* 2007).

Table 16. Survival of BB536 during refrigerator storage(4ºC) of different fermented barley beverages

	Fermented Beverages			
		Barley Beverages		
Storage (Week)	Re-constituted skim Milk	Bukur	Balady	Local 46
Initial (maximum growth)	8.13±0.01[a]	8.21±0.04[a]	8.08±0.05[a]	8.17±0.06[a]
First	7.79±0.07[a]	7. 89±0.02[a]	7.37±0.35[a]	7.40±0.27[a]
Second	7.56±0.34[b]	7.07±0.019[b]	6.63±0.07[b]	6.99±0.02[b]
Third	6.82±0.05[c]	7.07±0.019[b]	6.16±0.060[c]	6.60±0.050[c]

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

4.13. Changes in water content throughout the storage period of different fermented beverages

In Table 19 water content of different fermented beverages significantly ($p<0.05$) increased during the storage period for three weeks. the increasing due to release of water from the breakdown of macro components. The water content increased during the first week of refrigerated storage of different formulated fermented Re-constituted skim milk and Bukur, Balady, Local 46 barley beverages supplemented with the levels of increasing BB536 were 1.98, 2.03, 2.23 and 1.28%, respectively. While the increase in the third week of refrigerated storage were 3.67, 3.50, 3.39 and 2.07% for Re-constituted skim milk and Bukur, Balady, Local 46 fermented barley beverages respectively. In food, water content is essential for shelf life, it is used to predict microbiological and chemical stability of food products. In the fermentation process, increase in water content might indicate a high enzymatic activity that breaks down the macro-components into simpler ones and release of water.

4.14. Microbiological properties of fermented beverage barley during storage

The result presented in table 20 shows that there are significant ($p < 0.05$) differences in total viable count of bacteria regarding different fermented products during storage for three weeks including Re-constituted skim milk, Bukur, Balady and Local 46 fermented barley beverages. Yeast and mould, *Staphylococcus,* coliform*, E. coli, and Salmonella* were not found or detected in any of the developed fermented products over the storage period at 4°C. The absence of these pathogenic bacteria is due to the bifidogenic effect of strain BB 536 even after post fermentation. The strain is reported to produce antibiotic like components against the pathogenic bacterial strains. These results prove that all samples were processed, handled and stored under healthy condition, were

suitable for human consumption and in according to the microbiological standards required. This indicates that proper care was taken to avoid contamination throughout the process and the product has good safety standard. Salij and Saadi, (1986) found lower coliform bacteria in the fermented beverage. Steinkraus (2002) and Erbas *et al.* (2006) noticed that fermented food are safe due to low pH and high organic acids, such as lactic acid. Therefore, production of acid and other antimicrobial components during fermentation may promote or improve the microbiological safety and stability of the product (Holzapfel, 1997). The acid produced also lowers pH which slows down the rate of microbial spoilage and inhibits the growth of pathogenic organisms like coliforms (Steinkraus, 1996). Similar results were reported by Ifeanyi *et al.,* (2013) who found that initial counts of yeast and mold were not more than 1 CFU/mL in barley beta glucan enriched yoghurt. Elsanhoty *et al.* (2009) and Holzapfel and Schillinger (2014) stated that the coliforms were not detected all over storage period in yoghurt at the beginning and at the end of the storage periods.

Table 17. pH of the different fermented beverages during refrigeration Sorage (4°C)

Storage (Week)	Fermented Beverages			
	Re-constituted skim Milk	Barley Beverages		
		Bukur	Balady	Local 46
Initial(maximum growth)	4.18±0.005^{d}	3.57±0.005^{d}	3.55±0.010^{d}	3.60±0.005^{d}
First	4.09±0.02c	3.38±0.001^{c}	3.46±0.016^{c}	3.52±0.005^{c}
Second	3.95±0.005^{b}	3.26±0.001^{b}	3.33±0.01^{b}	3.44±0.001^{b}
Third	3.73±0.005^{a}	3.15±0.005^{a}	3.22±0.001^{a}	3.36±0.005^{a}

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

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Table 18. Total soluble solids (%) of the different fermented barley beverages during refrigeration (4ºC) storage

Storage (Week)	Fermented Beverages			
	Re-constituted skim Milk	Barley Beverages		
		Bukur	Balady	Local 46
Initial(maximum growth)	5.9±0.10[a]	4.00±0.10[c]	5.13±0.05[c]	4.03±0.20[c]
First	5.00±0.08[b]	5.06±0.16[a]	5.93±0.09[a]	5.78±0.06[a]
Second	4.47±0.02[c]	4.15±0.09[b]	5.86±0.09[b]	5.30±0.08[b]
Third	4.32±0.05[d]	3.95±0.10[c]	4.20±0.05[d]	4.60±0.20[d]

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

Table 19. Water content of the different fermented beverages during refrigeration storage(4ºC)

Storage Period	Fermented beverage			
	Re-constituted skim Milk	Barley varieties		
		Bukur	Balady	Local 46
Initial(maxium growth)	86.22±0.10[a]	86.28±0.01[c]	86.14±0.01[a]	86.45±0.29[a]
First	88.20±0.00[b]	88.31±0.12[b]	88.37±0.22[b]	87.73±0.02[b]
Second	89.26±0.00[c]	90.14±0.70[c]	90.14±0.02[c]	91.80±0.01[c]
Third	92.93±0.04[d]	93.64±0.02[d]	93.53±0.02[d]	93.87±0.02[d]

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column aren't significantly different at $p<0.05$.

4.15. Sensory evaluation

Sensory properties including colour, aroma, taste, odour and overall accaptacibility were considered. They are the most important properties for customer preferences. The sensory properties of fermented barley beverages were presented in Table (21). The result of sensory evaluation indicates that there were no significant differences ($P<0.05$) in aroma, taste and odour between the Re-constituted skim milk and other fermented barley varietiets beverages. The best colour and overall acceptability however were showed significantly differences ($p < 0.05$) in all types of fermented beverages. The best colour score was 1.44 which was excellent obtained in beveragere-constituted skim milk. While the lowest colour score was recored by Local 46 barley fermented beverage where was 3.22.

Table 20. Safety of strain BB536 fermented barley variety beverage during refrigeration storage(4°C)

Fermented beverages	Storage (week)	TVBC (log CFU/ml)	*E. coli*	coliform	*Salmonella*	*staphylococcus*	Yeast and moulds
Re-constituted skim milk	**First**	4.18±0.005[a]	Nill	Nill	Nill	Nill	Nill
	second	3.07±0.01[ab]	Nill	Nill	Nill	Nill	Nill
	Third	3.07±0.01[ab]	Nill	Nill	Nill	Nill	Nill
Bukur	**First**	4.11±0.00[c]	Nill	Nill	Nill	Nill	Nill
	second	3.06±0.015[b]	Nill	Nill	Nill	Nill	Nill
	Third	3.06±0.015[b]	Nill	Nill	Nill	Nill	Nill
Balady	**First**	4.17±0.00[b]	Nill	Nill	Nill	Nill	Nill
	second	3.08±0.01[a]	Nill	Nill	Nill	Nill	Nill
	Third	3.08±0.01[a]	Nill	Nill	Nill	Nill	Nill
Local 46	**First**	4.04±0.005[d]	Nill	Nill	Nill	Nill	Nill
	second	3.00±0.00[c]	Nill	Nill	Nill	Nill	Nill
	Third	3.00±0.00[c]	Nill	Nill	Nill	Nill	Nill

Values are mean ± SD for replicate independent runs.

Values that carry same superscript letters in the same column for each specifis type of beverage aren't significantly differentat $p<0.05$.

Table 21. Sensory score of fermented barley beverages

Type of fermented beverages	Characteristic				
	Flavor	**Color**	**Taste**	**Odour**	**Overally accaptacibility**
Bukur	2.77±0.97[a]	1.44±0.72[b]	2.78±1.20[a]	1.80±0.66[a]	2.33±0.87[ab]
Balady	2.44±1.33[a]	2.00±1.12[b]	2.66±1.00[a]	1.66±0.70[a]	2.44±0.9[ab]
Local 46	1.90±1.05[a]	3.22±0.97[a]	3.00±0.70[a]	2.44±1.13[a]	3.00±0.70[a]
Re-constituted skim milk	1.77±0.83[a]	1.44±0.75[b]	1.87±1.00[a]	1.56±0.52[a]	1.87±0.78[b]

Mean±SD

Values that bear different superscript letter in the same column are significantly different at $p<0.05$.

Scoring results of the four fermented beverage samples based on five-point hedonic scale ratings, where 1 = like extremely, 2 = like very much, 3 = like moderately, 4 = like slightly, 5 = dislike.

4.16. Effect of oral feeding on body weights of rat

Table 22 presented the weight gains of rats during the experimental period of 30 days. The initial weight was measured before starting treatment and there was no significant difference between the five groups of rats.The average of initial body weight recorded a range of 81.33-113.17g for C and M groups, respectively. The body weight increased gradually in all groups, recording 131.83 g in the control group and 168.17g for the B group. Rats fed fermented re-constituted skim milk and fermented barley beverages (A, B and C) gained highest weights as compared to control groups. All groups of rats have showed an equal growth pattern with variances in weight gain ranged between 42.33 and 75.00g at the end of experimental treatment period (Table 22). Therefore, this preliminary results point toward the safe profiles of *Bifidobacterium* fermented barley beverage fed rats. Usually in animal model studies, loss of weight is sign of negative health effects. The losses may take place as a result of toxicity or illnesses (Abdo *et al.,* 2001). Miyamoto *et al.* (2018) found that barley flour containing high levels of beta glycan was efficacious against weight gain by reducing food intake and improving insulin sensitivity. Similarly, Al-rewashdeh(2009) reported that rat groups fed barely and wheat containing diets had lower daily food intake than those fed with the control diet.This effect might be due to higher levels of phenolic compounds and β-glucan. Of these, β-glucan is recognized as an anti-obesity factor. Phenolic compounds in cereal have also been shown to have a suppressive effect on induced obesity, further suggesting that those in barley are primary anti-obesity mediators.This lower food intake was reflected by a significantly lower mean body weight gain. modulation of gut microbiota by probiotic treatment or dietary intervention lead to beneficial effects on body weight, influencing on glucose and fat metabolism, improving insulin sensitivity and reducing chronic systemic inflammation (Tennyson and Friedman, 2008).

4.17. Feed intake and water consumption of rats orally fed with different treatments

The results of average feed and water consumption in control and experimental groups of rats during the 30 days of study period are presented in Table 23. The average feed intake of rat groups (control, M, A, B and C) ranged between 87.39 and 105.19 g at the end of the first week. Also, the results in Table (23) showed that there were no significant differences ($p > 0.05$) between five different rats groups in feed consumption. The lowest feed consumption was in the group A. Moreover, the highest feed consumption was recorded for group B orally fed fermented Balady barley beverage. Water is important to the health and makes up approximately two-thirds of the body by weight. In orderto maintain physical balance in the body, it is necessary to coordinate and integrated link between the various organs. Al-Rewashdeh (2009) found that Rats seemed to eat less after feeding high satiety diets (fiber containing diets) than after feeding low satiety diet (fiber free diet). Also, Kalra and Jood (1998) reported that a proportion of the hull content of barley affects the feed intake of rats. It was also reported that b-glucan pre- parations from different cereals affected weight and feed efficiency of rats. Dietary fiber has a high water holding capacity (Eastwood, 1973) and it is likely that rats fed fibers, in spite of the lower energy in their diet compared to those fed fiber- free diet, did not eat more because the fiber is swelling in their intestine and gives satiety sense. As a result rats fed fiber diets utilized more fats from their food, while the control rats deposited it in their tissues.

4.18. Haematological parameters of rats orally fed with different fermented barley beverages

Haematological parameters of the rats fed fermented barley beverage is as shown in Tables 24, there is no any indication of any health problems or serious illness symptoms on rats group fed by fermented barley product. In addition no signs of

any deviationd or deficiencies in CBC parameters were recorded. Further there areconsiderable signs of positive health effect on red blood cells (RBC), haemoglobin (HGB) when compared with the control rat group. Red blood cells (RBC), (HGB) 8.40(10^6 u/L), is much higher in rat groups fed Bukur barley fermented beverage followed by group fed Recondstited skim milk fermented beverage 8.37(10^6 u/L). HCT, MCV,MCH/pg, RDW-SD%, PLT levels, MPV, PCT,Monocyte, Eosinophil and Bosinoph of rat groups were within the standard range and were not significant ($p > 0.05$) between the all groups of rats.While there were significant differences ($P > 0.05$) increase in the (HGB and Lymph) in all treatments except group (B). Neutrophil and platelet count were increased in all groups compared with control.Variation in CBC parameters may be associated with each individual rat adaptability to environmental conditions as stated by Koubkova *et al*,(2002). Fermented barley beverage supplemented with *Bifidobacterium longum* BB536 oral feeding did not produce any abnormal change effect on haematological parameters. Therefore, increased of lymphocytes count (lymphocytosis) is an indication that fermented oral feeding barley beverage led to improved immune system. While the reduced monocytes suggest absence of contamination. In general there are good indications of inflammation and toxicity absence in rat groups. This result is inagreement with previous reported by Yiming *et al*. (2012) on lymphocyte proliferation and immune modulatory properties of the spices. Moreover, all the changes that observed in hematological parameters were within normal standard ranges of rats.

4.19. Blood biochemistry parameters of rats orally fed with different fermented barley beverages

The effect of feeding different fermented barley beverages on blood biochemistry parameters of rats is presented in Table 25. The glucose level of rats orally fed

fermented barley beverage was lower than that of the control rats without fermented product. It might be due to the Barley content of β-glucan which has various physiological effects on regulating blood glucose level and insulin response among diabetics (Cavallero *et al.,* 2002). Behall *et al.* (2005) reported both oat and barley can reduce glycemic responses, decrease glucose level and insulin responses as a result of the high soluble fiber content of them which considered as major factor. Total protein level was higher in (M and A) followed by B, C and control rat group. The concentration of globulin was not significantly ($p<0.05$) different but the albumin was clear between rats groups. Electrolytes play vital role in keeping body fluid pH and the dynamic equilibrium of water (Radostits *et al.,* 2007). Sodium, potassium, calcium, phosphate concentrations in the blood of treated rats groups were significantly ($P<0.05$) different. Also, creatinine showed significant ($p<0.05$) differences between all treated groups of rats. While uric acid did not significantly different in all groups. creatinine, potassium ion and sodium high level in blood indicated kidney damage. In this study, serum electrolytes in all treated rats group were showed significant ($P< 0.05$) differences as compared with the control group. Nevertheless, all values were within the normal ranges of rats.Evaluation of liver functions is very important in toxicity assessment because organs are necessary for the endurance and welfare of an organism. Referring toTable 25, AST and ALT did not showed significantly ($P< 0.05$) different change as compared with the control. D. Billi and ALP were significantly ($P< 0.05$) different between rat groups. However, any decreasing in the level of serum ALT, AST, and ALP activity are indication of hepatic disease (Henry, 2003). ALT may produced by other tissues such as the heart, skeletal muscle and kidney, but more specific in liver and thus it is used as a reliable marker for liver specific condition. AST also is an enzyme used in liver assessment but it is less specific. It is predominant in the heart tissue, as well as

in the skeletal and renal tissues (Chernecky and Berger, 2008). ALP is an enzyme found in bone, liver, intestine, and placenta increasing in its activity in blood that mean under lying conditions of these tissues. Factors such as biliary tree obstruction elevate ALP by the hepatocytes adjacent to the biliary canaliculi, which then leaks into plasma (Burtis *et al.*, 2008). In this study all levels of blood enzymes were within the normal standard range of rats. All groups orally treated with fermented barley beverage showed best serum activity, due to their high phenolic compounds and beta glucan content. The results of the biological assay in Table 25 show the changes in plasma HDL LDL, cholestrol and triglyceride levels at the end of experiment. HDL and LDL (A, B, and C) in orally fed rat reduced serum triglyceride, total cholesterol, as compared with the rat control group. The blood cholesterol and triglyceride levels in Table 25 shows that all the values (100, 78.33, 84.00, 80.67 and 84.00 (mg/ dL) for cholesterol and 113.67, 107.00, 74.00, 98.67, 102.62 and 93.00 (mg/ dL) for C, M, A, B and C, respectively. The rat groups fed the fermented barley beverages recorded the lowest level in cholesterol, and triglyceride as compared with the control group. These findings were consistent with those of several studies reported such as, Jue *et al.*(2004), who reported that the barley diet significantly decreased the plasma levels of total cholesterol, triacylglycerol, and LDL-cholesterol. However, Sindhu and Khetarpaul, (2003) found that the rats group fed by probiotic fermented diet resulted significant decreases in total serum cholesterol and triglycerides, whereas, HDL cholesterol increased compared to control group diets. On the other hand, Beena and Prasad, (1997) found lower serum cholesterol and LDL-cholesterol in rats fed on yoghurt containing *Bifidobacterium bifidum* compared to a positive control after 30 days. Kikuchi-Hayakawa *et al.* (1998) and Abd El-Gawad *et al.* (2005) reported that consumption of *bifidobacterium* fermented soymilk significantly reduced plasma cholesterol VLDL+LDL-

cholesterol and increased the HDL-cholesterol level in animals fed cholesterol-enriched diet. Abd El-Rahman (2000) suggested that the yoghurt diets supplemented with *B. bifidum* reduced cholesterol in rat liver tissues. Kikuchi-Hayakawa *et al.* (1998) found that the total cholesterol in hamsters fed on a cholesterol-free diet, soymilk or soymilk fermented with *B.breve*, were lower than that in the control hamsters fed on basal diet. Liong and Shah, (2006) stated that probiotics are capable of inhibiting intestinal cholesterol absorption by binding bile acids and cholesterol. Barley has a concentration of soluble fibers called β-glucan, which associated with increased excretion of bile acids and neutral sterols, increased catabolism of cholesterol, and reduced absorption of cholesterol and fat (Talati *et al.,* 2009). Barley may be used as a part of the vegetarian diet because it decreases serum total lipids. Moreover, whole-grain barley is higher in total dietary fiber it is increasing whole-grain consumption can reduce the risk of coronary heart disease, and keep weight maintenance (Jenkins *et al.,* 2003). Among the beneficial effects attributed to probiotics and probiotic containing food products, the reduction of blood cholesterol is of particular interest (Cavallini *et al.,* 2009). Our results are in agreement with study conducted by Joanne *et al.*(1994), who reported that β-glucan reduced lipids and cholesterol in rats. Fukushima and Nakano, (1995) reported decreasing in hepatic cholesterol concentration in rats fed on probiotic such as *Bifibacterium* fermented foods. Several mechanisms are suggested for cholesterol reducing activity of probiotics. One of them is deconjugating bile acids through bile salt hydrolase catalysis. Since, cholesterol is the precursor for the synthesis of new bile acids the use of cholesterol, to synthesize new bile would lead to decreasing concentration of cholesterol (Lye *et al.,* 2010). For taking up and assimilating cholesterol for their cell membrane and binding cholesterol to cell walls of probiotics in intestine (Tanaka *et al.,* 1999), conversion of cholesterol into

coprostenol (Lye *et al.,* 2010) and short chain fatty acids such as propionate produced by probiotic bacteria may also inhibit hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to the liver (Wolever *et al.* 1996), It has hypocholesterolemic effect via altering the path ways of the cholesterol esters and lipoprotein transporters (Oi and Liong, 2010).

Table 22. Weight (g) of different groups of rats orally fed with different fermented beverages for 30 days

Weight (g)	Oral feeding groups				
	Control	M	A	B	C
Initial body weight	93.17 ±16.86^{b}	113.17±12.5^{a}	108.67 ±8.14^{ab}	100.50±16.16^{ab}	81.33 ±9.00^{a}
Final body weight	131.83±12.73^{c}	155.50±7.06^{ab}	148.50± 9.25^{bc}	168.17±16.15^{a}	160.50± 8.07^{ab}
Weight gained	38.66±10.17^{ab}	42.33± 24.8^{a}	39.83±21.08^{b}	59.83± 8.76^{b}	79.17±5.19^{ab}

Values are means ±SD (n=6); Means with the same superscripts letters in each colum are not for each specific group at initial and final body weight significantly different ($p < 0.05$).

Values are means ±SD (n=6); Means with the same superscripts letters in each raw are not for each specific group weight gain significantly different ($p < 0.05$).

Control group: orally fed sterile water.

M: orally fed *bifidobacterium longum* BB536 fermented re-constitend skim milk.

A: orally fed *bifidobacterium longum* BB536 fermented barley beverage Bukur variety.

B: orally fed *bifidobacterium longum* BB536 fermented barley beverage Balady variety.

C: orally fed *bifidobacterium longum* BB536 fermented barley beverage Local 46 variety.

Table 23. Feeding intake (g/day) and water consumption (ml/day) of rat groups fed orally with different beverages

Parameter	Oral feeding groups				
	Control	M	A	B	C
Food intake	91.8 ± 55.7^{a}	88.57 ± 44.8^{a}	87.39 ± 36.8^{a}	105.13 ± 39.7^{a}	91.26 ± 36.3^{a}
Water consumption (ml/day)	191.74 ± 40.24^{ab}	236.1 ± 74.1^{bc}	135.22 ± 31.82^{a}	121.39 ± 32.5^{c}	158.1 ± 87.8^{c}

Values are means ±SD (n=6); Means with the same superscript letters in each row are not significantly different ($p \leq 0.05$).

Control group: orally fed sterile water.

M: orally fed *bifidobacterium longum* BB536 fermented re-constitend skim milk.

A: orally fed *bifidobacterium longum* BB536 fermented barley beverage Bukur variety.

B: orally fed *bifidobacterium longum* BB536 fermented barley beverage Balady variety.

C: orally fed *bifidobacterium longum* BB536 fermented barley beverage Local 46 variety.

Table 24. Haematology parameters of rats orally treated with different fermented barley beverages for 30 days

Parameters	Treatments				
	Control	**M**	**A**	**B**	**C**
WBCs /L	5.45±0.35^{b}	5.46±1.00^{b}	8.25±0.65^{a}	6.30±1.32^{ab}	4.80±1.0^{b}
HGB g/dl	13.16±0.15^{c}	15.17±0.55^{a}	14.90±0.60b^{a}	13.26±0.80^{bc}	14.70±0.45^{ab}
RBCs/L	7.41±0.16^{b}	8.37±0.45^{a}	8.40 ±0.30^{a}	7.37 ±0.37^{b}	8.15±0.32^{ab}
HCT %	42.40±3.20^{a}	47.37±1.97^{a}	46.27±1.97^{a}	42.00±1.70^{a}	45.167±1.52^{a}
MCV/fl	55.267±1.20^{a}	56.70±0.72^{a}	55.167±0.66^{a}	57.033±0.64^{a}	55.57±2.00^{a}
MCH/pg	17.73±0.20^{a}	18.06±0.35^{a}	17.70±0.17^{a}	17.93±0.60^{a}	18.03±1.10^{a}
MCHC/g/dl	32.13±0.32^{a}	31.96±0.379^{a}	32.13±0.15^{a}	31.53±1.25^{a}	32.53±1.00^{a}
RWD Cv/%	15.10±1.15^{a}	14.00±1.05^{a}	14.30±0.17^{a}	15.90±0.43^{a}	15.06±1.36^{a}
RDW-SD/%	31.07±2.22^{a}	29.90±0.52^{a}	30.50±0.00^{a}	33.067±1.44^{a}	31.36±0.85^{a}
PLT/L	666.0±27.5^{bc}	768±25.0^{abc}	646.7±39.5^{c}	815.7±57.5^{ab}	746.7±52.5^{a}
MPV/fl	6.63±0.25^{a}	6.73±0.35^{a}	7.03±0.15^{a}	6.80±0.45^{a}	6.93±0.25^{a}
PDW/fl	15.96±0.15^{a}	15.36±0.152^{b}	15.60±0.173^{ab}	15.40±0.172^{b}	15.70±0.20^{ab}
PCT%	0.45±0.04^{a}	0.53±0.09^{a}	0.48±0.07^{a}	0.55±0.03^{a}	0.49±0.10^{a}
Neutrphili%	56.33±2.52^{a}	49.67±8.33^{ab}	46.66±0.57^{ab}	37.66±1.52^{b}	46.67±6.03^{ab}

Lymph%	29.50±1.50^{a}	43.00±3.00^{b}	37.00±1.00^{c}	53.00±1.00^{d}	40.66±1.15^{bc}
Monocy%	5.00±1.73^{a}	8.00±1.73^{a}	6.67±2.52^{a}	7.33±1.15^{a}	9.33±1.15^{a}
Eosinophilis%	5.00±5.29^{a}	6.33±1.52^{a}	2.66±1.52^{a}	2.33±4.04^{a}	3.33±0.577^{a}
Bosinophilis%	0.667±0.057^{a}	0.33±0.057^{a}	0.00±0.00^{a}	0.00±0.000^{a}	0.00±0.00^{a}

Values are means ±SD (n=6); Means with the same superscript letters in each row are not significantly different ($p \leq 0.05$).

Control group: orally fed sterile water

M: orally fed *bifidobacterium longum* BB536 fermented re-constitend skim milk.

A: orally fed *bifidobacterium longum* BB536 fermented barley beverage Bukur variety.

B: orally fed *bifidobacterium longum* BB536 fermented barley beverage Balady variety.

C: orally fed *bifidobacterium longum* BB536 fermented barley beverage Local 46 variety.

Table 25. Blood biochemistry profile of rats orally fed different fermented barley beverage for 30 days

Parameters	Treatments				
	Control	M	A	B	C
Glucose mg/dl	110±3.61^{c}	105±5.57^{a}	100.33±6.66^{b}	97.00±4.00^{bc}	93±3.61^{ab}
Total protien/g/dl	6.86±0.05^{a}	7.00±0.10^{a}	7.00±1.56 ^{a}	6.96±0.020^{a}	6.90±0.52^{a}
Albumin g/dl	2.86±0.28^{a}	2.56±0.28^{a}	2.633±0.40^{a}	2.76±0.11^{a}	2.66±0.37^{a}
Globulin g/dL	4.06±0.11^{a}	4.43±0.15^{c}	3.66±0.11^{ab}	4.36±0.15^{b}	4.733±0.15^{ab}
Sodium mmol/l	139.12±0.01^{a}	135.66±0.01^{c}	141.75±0.87^{b}	139.42±0.50^{b}	139.16±0.05^{b}
Potassium/mmol/l	2.56±0.011^{a}	2.55±0.017^{b}	2.83±0.02^{b}	2.54±0.03^{b}	2.71±0.13a^{b}
Calicium /mg/dl	7.10±2.43^{a}	12.40±1.03^{b}	8.73±0.47^{ab}	9.80±1.05^{ab}	9.73±1.50a^{b}
Phosphor /mg/dl	4.36±0.04^{a}	5.28±0.051^{e}	4.12±0.020^{c}	4.51±0.005^{d}	4.72±0.043^{b}
blood urea/mg/dl	47.00±0.00^{a}	48.33±0.57^{c}	47.00±0.00^{b}	49.00±1.00^{b}	43.00±1.00^{ab}
Creatinine /m g/dl	0.43±0.05^{a}	0.50±1.00^{b}	0.56±0.05^{ab}	0.66±0.05^{ab}	0.53±0.05^{ab}
Uric acid mg/dl	4.60±0.10^{a}	4.80±0.56^{a}	4.06±0.11^{a}	4.73±0.57^{a}	4.16±.15^{a}
D.Billi (mg/dl	0.63±0.05^{a}	1.2±0.15^{c}	0.60±0.10^{bc}	0.93±0.057^{c}	0.70±0.10^{b}
AST/U/L	133.67±6.51^{a}	169.0±18.0^{b}	152.0±18.0^{b}	151.7±25.0^{b}	209.67±13.50^{ab}
ALT/U/L	78.00±8.19^{a}	90.33±5.03^{ab}	70.00±6.24^{ab}	76.67±5.13^{ab}	76.33±4.73^{ab}

ALP/U/L	95.67±4.12^{c}	118.00±0.55^{ab}	112.43±2.18^{ab}	125.54±5.32^{a}	109.02±4.20^{ab}
HDL/mg/dl	34.60±7.19^{b}	45.50 ± 3.10^{a}	34.60± 0.96^{b}	34.80±3.24^{ab}	39.90±2.65^{ab}
LDL/L/mg/dl	19.30± 3.21^{a}	31.77±5.97^{a}	29.67 ±5.05^{a}	23.13±2.42^{a}	25.50 ± 6.95^{a}
Chlosterol /mg/dl	100.00±7.21^{a}	78.33±2.52^{b}	84.00±6.56^{b}	80.67±3.21^{b}	84.00±5.29^{b}
Triglusride/mg/dl	113.67±4.73^{a}	107.67±2.08^{ab}	98.67± 11.02^{ab}	113.67±10.97^{a}	93.00±4.58^{b}

Values are means ±SD (n=6); Means with the same superscripts letter in each row are not significantly different ($p \leq 0.05$).

Control group: orally fed sterile water

M: orally fed *bifidobacterium longum* BB536 fermented re-constitend skim milk.

A: orally fed *bifidobacterium longum* BB536 fermented barley beverage Bukur variety.

B: orally fed *bifidobacterium longum* BB536 fermented barley beverage Balady variety.

C: orally fed *bifidobacterium longum* BB536 fermented barley beverage Local 46 variety.

4.20. Microbiology of rats

Table (26) presented Different microbial groups in colon of rats. Total aerobes in the large bowel changed little due to the ingestion of fermented barley beverages as compared with the control. Total aerobic in colon were significant ($p<0.05$) different increase between all groups orally of rats received the different fermented barley beverages the control. Group (A) showed the highest increase in total number aerobes of colon (7.92 log CFU/ g) whereas, group(C) level of 6.96 log CFU/ g. In general, total anaerobes were also higher in rats fed with barley beverage compare with control, but was lower in group C. Although prebiotics offer one rational approach to the probiotic concept, the health consequences have not yet been defined. However, the *bifidobacteruim* and lactic acid bacteria of the gastrointestinal tract are thought to play a significant role in improveing colonization resistance so fermented barley beverage with *bifidobacterium* led to increasesin number of lactobacillus inside colons of rats in all group as compared with the control. Bifidobacteria displayed the highest increased among all microbiota communities in colon is fine.The increases were significant ($P <0.05$) between rat groups. Increased bifidobacterial numbers in the gut may be one factor that contributes towards improved competitive exclusion of pathogens. Also, Probiotics have numerous advantageous functions in organisms, their maintain proper balance between pathogenic and normal flora bacteria in gut (Oelschlaeger, 2010). Moreover, many lactobacilli and bifidobacteria are able to produce natural antibiotics, which have a broad spectrum of activity against various intestinal pathogens (Shiba *et al*., 2003). Lactobacillus and Bifidobacterium are non-pathogenic bacteria of the bowel tract. These micro-organisms may increase resistance to disease by reducing the growth of pathogenic and putrefactive bacteria by producing inhibitory

substances, competing directly for substrates and mucosal attachment sites (Jacobsen *et al*., 1999).The harmful pathogens of gut microbiota contain species such as *Coliform, Salmonella* and *Staphylococcus*, Table (26) showed that all pathogenic were significant ($p<0.05$) different decreased in all groups orally within fermented products treated compared with control. The coliform was not found in colon of group Arats, but decreased in other groups. *Salmonella* was not found in colon of groups A and, C rats. This result might be affected of *bifidobacterium* and antioxidant activity of barley compounds. *Staphylococcus* decreased in groups D followed by B, C and A. These results showed that fermented barley beverages may have viable affected on recrding growth of the harmful pathogens in colon of fed rats. Therefore, probiotics may effectively inhibit the development of pathogenic bacteria, such as *Clostridium perfringens*, *Campylobacter jejuni*, *Salmonella Enteritidis*, *Escherichia coli*, various species of *Shigella*, *Staphylococcus*, and *Yersinia*, so prevent us from food poisiong (Schoster *et al.,* 2013). Also, Helland *et al*. (2004) reported that the probiotic bacteria have a variety of positive side impact on advantage on different mechanism by into fining in physiological pathways.

Table 26. Microbial groups (Log CFU/g) from colon of rats received different fermented barley beverages

Bacterial groups (log CFU/g)	Treatments				
	control	M	A	B	C
Total anaerobe	7.05±0.71^{ab}	7.72± 0.29^{a}	7.92±0.02^{a}	7.07±0.04^{b}	6.96±0.85^{b}
Total aerobe	7.43±0.56^{ab}	7.81±0.02^{ab}	8.49 ±0.65^{a}	7.50 ±0.40^{a}	8.60±0.55^{a}
Lactobacillus	5.15±0.03^{c}	7.81±0.05^{a}	8.11± 0.01^{a}	7.59 ±0.47^{a}	6.54±0.47^{b}
Bifidobacteria	5.58±0.47^{b}	7.03±0.02^{a}	6.86± 0.04^{a}	7.77±0.04^{a}	7.23± 0.04^{a}
Coliform	5.87± 0.04^{a}	3.21±0.10^{d}	0.00±0.00^{e}	3.84±0.04^{b}	3.52±0.03^{c}
Salmonella	4.86±0.05^{ab}	3.73±0.03^{b}	0.00±0.00^{c}	3.93± 0.12^{b}	0.00±0.00^{c}
Staphylococcus	4.07±0.06^{ab}	4.28±0.48^{ab}	4.70±0.15^{a}	4.46±0.47^{ab}	3.73±0.27^{b}

Values are means ±SD (n=6); Means with the same superscripts letter in each row are not significantly different ($p \leq 0.05$).

Control group: orally fed sterile water

M: orally fed *bifidobacterium longum* BB536 fermented re-constitend skim milk.

A: orally fed *bifidobacterium longum* BB536 fermented barley beverage Bukur variety.

B: orally fed *bifidobacterium longum* BB536 fermented barley beverage Balady variety.

C: orally fed *bifidobacterium longum* BB536 fermented barley beverage Local 46 variety.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

1. Malting process lead to improved nutritional value (fiber, carbohydrate, and total sugars) of the different barley varieties.
2. The developed barley mediums is cheap and contribute positively to deliver *Bifidobacterium longum* BB536 at recommended level of probiotics foods at recommended level of probiotics foods.
3. Fermented barley beverage exerted probiotic effect in *Vivo* levels (experiment on Albino rats).
4. Fermented barley beverage has a positive effect on glucose metabolism and reduction of body weight which could be useful for obesit.
5. Feeding fermemted barley beverages exerted a positive health effect (the weight gian of fed rats, no sings of toxicity or pathogenicity revealed on blood hematology and biochemistry), thus it is safe for human consumption.

5.2. Recommendation

1. Encourage planting of barley in Sudan since; it contains nutritional value and antioxidants.
2. More research should be conducted on optimizing the effects of malting on nutritional quality of barley
3. Further improve growth and survival of different strains of probiotic in barely beverages.
4. Improve palatability and other sensory characteristics of different probiotic fermented barley beverages for scaling up purpose.

5. More research to be conducted to explore curing effect of fermented barley against diseases such as diabetes and cardiovascular.

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Appendixes

appendix 1. Soaking of barley

Appendix 2. germinanting of barley

Appendix 3. Albino Rats