Int.J.Curr.Microbiol.App.Sci (2020) *9*(10): 2109-2118

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 10 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.910.257

Probiotic *Bifidobacterium longum* BB536 Viable Existence at Refrigeration Storage of Fermented Goat Milk Supplemented with Inulin and Different Cereal Bran (Sorghum, Barely and Millet)

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Keywords

Bifidobacterium, Goat milk, Cereal bran, Existence, Refrigeration

Article Info

Accepted:
17 September 2020
Available Online:
10 October 2020

A B S T R A C T

This study was carried out to evaluate viable existence of *Bifidobacterium longum* BB536 during two weeks refrigeration of fermented goat milk supplemented with inulin and different cereal bran (Sorghum, Barely and Millet). Fermentation mediums were formulated from goat milk supplemented with 10% inulin and different cereal bran (sorghum, barely and millet). Probiotic strain *B. longum* BB 536 was used as the starter culture for 12h incubation to attain the fermented products. Two weeks refrigeration period was design for the fermented products. Different analyses including: strain BB536 viable count, reducing sugar, physicochemical analysis (TSS, pH, acidity) and moisture were conducted. The maximum viable existence of strain BB 536 throughout refrigeration (two weeks) was in fermented goat milk supplemented with millet bran (lowest reduction of 1.48CFU /ml); whereas, the best existence in the first week was in fermented goat milk supplemented with barely bran (0.76 CFU /ml). Therefore, the strain existence trend was dependent mainly on both type of fiber source and refrigeration period. Hopefully, the final viable count of strain BB536 in all products was above the minimum number required to presence in probiotic to exert health benefits upon consumption. During refrigeration of fermented products reductions in reducing sugar, TSS and pH; and increases in acidity and moisture were revealed due to the slight strain BB536 enzymatic activities. Therefore, maximum viable existence of strain BB536 in fermented goat milk under refrigeration for two weeks could be achieved by supplementation with millet bran; while better existence in the first week was attainable with barley bran supplementation.

Introduction

The word ‘probiotic’, derived from the Greek language, means ‘for life’ (Fuller, 1989) and has had many definitions in the past. Definitions such as ‘substances produced by protozoa that stimulate the growth of another’ or ‘organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance’ were used (Fuller, 1989). These general definitions were unsatisfactory

because 'substances' include chemicals such as antibiotics. The definition of probiotics has since then been expanded to stress the importance of live cells as an essential component of an effective probiotic. Furthermore, Huis-Veld and Havenaar (1991) broadened the definition of probiotics as being 'a mono- or mixed culture of live microorganisms which, applied to man or animal (e.g. as dried cells or as a fermented product), beneficially effects the host by improving the properties of the indigenous microflora. This definition implies that probiotic products, for example fermented milk, contain live microorganisms and improve the health status of the host by exerting beneficial effects in the gastrointestinal tract.

Bifidobacterium longum is one of the *Bifidobacterium* species found mainly in human feces and it considered as the most common species being found both in infant and adult. Potential benefits from consumption of *B. longum*BB536 include: antagonistic action toward intestinal pathogens, improved lactose utilization, anticarcinogenic action and control of serum cholesterol levels (Kojima *et al.,* 1996; Namba *et al.,* 2003). Thus, there is still considerable interest in incorporating this probiotic *Bifidobacterium* into food. Nevertheless, probiotic strains, particularly *Bifidobacterium* are rarely used outside the diary based industry. Together with the scarcity of animal milk in many countries makes it difficult to provide adequate intake of this health promoting probiotic bacteria..

Milks contain, with some exceptions, the nutrients required for the growth and development of the neonate as well as probiotic bacteria. It contains specific proteins, fats designed to be easily digested, most have lactose, minerals, vitamins, and other components which may have important roles in supporting probiotic growth (Jensen *et al.,* 1991).

Goat milk differs from cow or human milk by having better digestibility, higher alkalinity, increased buffering capacity, and certain therapeutic effects that may be useful in medicine and human nutrition. The good acceptability and digestibility of goat milk are important beneficial factors for its inclusion in formulated diets prescribed for children and convalescent people. In many cases, goat milk may be successfully used as substitute for cow milk in the regular diet of allergic individuals (Haenlein, 2003). Thus, fermentation of goat milk with probiotics could further improve its therapeutic properties.

Dietary fibers are part of the plant cell which cannot be digested by the human enzymes. Two various groups of dietary fibers are recognized: soluble and non-soluble dietary fiber. They are remarkable by their solubility in water and show different physiological effects. The benefit of dietary fiber for a healthy diet is widely known. Different diseases, such as constipation, coronary heart diseases and cancer have been correlated to an unhealthy diet, low in dietary fiber (Kohimeler *et al.,* 1993). Dietary carbohydrates like resistance starch, insoluble fiber and soluble fiber that are able to stimulate, specifically the growth of potentially beneficial bacteria, e.g., bifidobacteria at the expense of the more harmful pathogenic microorganisms, are called prebiotics (Kouane *et al.,* 2005). On the other hand, dietary fibers are often characterized by high nutritional quality, as they are able to cure many chronic diseases and improve texture, sensory characteristics, and shelf life of foods. The fast growing food industry will likely generate an ever-growing amount of byproducts including bran, husk, peel, pomace, and other products that are rich

in dietary fibers (Betoret *et al.,* 2011). Recently, prebiotic effect of different cereal bran (sorghum, barely and millet) on growth of *Bifidobacterium longum* BB536 during fermentation of goat milk was approved (Mohamed *et al.,* 2020). However, sightseen of their roles during the storage are lacking. Therefore, the objective of this study is to evaluate the existence of strain *BB536* in fermented goat milk supplemented with cereal bran and assess its related physiochemical changes during refrigeration storage.

Materials and Methods

Raw Materials

Inulin was obtained from A natural Product Company (London, England). Different cereal bran (sorghum, barley and millet) were purchased from a local crops market at central market in Bahri (Khartoum state, Sudan). Goat fresh milk was obtained from the animal farm at Department of Animal Production, College of Agricultural Studies, Sudan University of Science and Technology (Khartoum, Sudan).

Preparation of cereal bran

Different cereal brans were ground and sieved using appropriate mesh. The resulting powder stored in a dark polyethylene bag at freezer until used.

Preparation of fermentation inoculums

B. 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 ^{o}C in 20% glycerol solution. Stock culture was prepared by activation of the strain in skim milk, incubation an aerobically at 37 ^{o}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 successive transformations of stock culture in 10% sterilized skim milk (121^{o}C for 15 min) and incubation at 37 ^{o}C for 24h.

Growth medium and fermentation conditions

Growth medium were formulated from goat milk supplemented with 1% inulin or different cereal bran (sorghum, barely and millet). Formulated medium were sterilized (121˚C for 15 min) and inoculated with a 3% active culture working of *B. longum BB536* followed by incubation at 37 ^{o}C for 24h.

Enumeration of viable *B. longum BB536* cell

MRS medium was used to enumerate *B. longum BB536* of different fermented products using the plate count technique. Fermented samples were drawn at initial and every 6h intervals during fermentation.1ml of fermentation broth was diluted in peptone water, followed by plating on Demann Rogosa agar (MRS) supplement with 0.05% L-cystiene. The plates were incubated unaerobically at 37 ^{o}C for 48 h. The growth was calculated as Colony Forming Unit per ml (CFU/ml).

Determination of reducing sugars

Ten gram of sample was weighted 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 milliliters of sugar solution was transferred into a conical flask containing 10 ml Fehling's solution representing 5 ml of Fehling A (6.928 gm CuSo4.5H2O per 100ml distilled water) and 5 ml Fehling B (34.6 sodium potassium titrate

and 10 gm NaOH per 100 ml distilled water) mixed well and then heated moderately to boiling 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, then reducing sugar was calculated following Schneider *et al.,* (1982) method.

Determination of titrable acidity

The titrable acidity (TA) of the different fermented products was determined according to AOAC method (2006). Ten ml of sample were weighted into a conical flask. Adistilled 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 pipetted into porcelain dish, five drops of phenolphthalein added, and the sample was titrated against 0.1N NaOH till a faint pink colour that lasted for at least 30 seconds was obtained; then acidity of different products was calculated.

Determination of total soluble solids (TSS)

Total soluble solids (TSS) of the fermented products were determined at room temperature using digital refractometer with degree Brix^{o} scale 0-100 according to AOAC (1990) method.

Determination of pH value

The pH value of the different fermented products 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 products samples was directly measured.

Determination of moisture content

Moisture was determined according to the modified method of AOAC (1990). Five grams of the sample was weight using in sensitive balance, after weighting the empty dishes and then 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 dessicator and allows to cool to room temperature before reweighting to calculated moisture.

Statistical analysis

One- way an ANOVA test was performed to examine significant differences between normally distributed data of replicated independent runs. Probability level of less than 0.05 was considered significant ($p<0.05$). All data were analyzed using vision 17 MINITAB statistical software for windows (2010).

Results and Discussion

The viable counts of *Bifidobacterium longum* BB536 log (CFU/ ml) during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Table 1 shows the viable counts of *B. longum BB536* during refrigeration storage of different formulated goat milk supplemented with inulin and different cereal bran (Sorghum, Barely and Millet). The survival of probiotic bacteria in fermented dairy bio-products depends on such varied factors as the strains used, interaction between species present, culture conditions, chemical composition of the fermentation medium (e.g. carbohydrate source), final acidity, milk solids

content, availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen (especially for *Bifidobacterium* sp.), level of inoculation, incubation temperature, fermentation time and storage temperature. Referring to the result in Table 1, there were significant ($p<0.05$) reductions in *B. longum BB536* viable count in all fermented samples at refrigeration. With regard to total reduction of strain BB536throughout the storage period (two weeks), the highest value was in goat milk supplemented with barley bran (1.92 CFU /ml), followed by sorghum bran(1.83 CFU /ml), inulin (1.81 CFU /ml)and then millet bran (1.24 CFU /ml) in descending order (Table 1). The maximum rate of reduction in the first week of the refrigeration storage was in in fermented goat milk supplemented with millet bran (1.48CFU /ml); while the lowest was in in fermented goat milk supplemented with barely bran (0.76 CFU /ml). Nevertheless, the trend of reduction based on fiber source differed in the second week. It was revealed highest strain BB536 reduction in goat milk supplemented with sorghum bran (1.57CFU /ml), but the lowest reduction was in goat milk supplemented with millet bran (0.44CFU /ml) as presented in Table 1. Therefore, the trend of strain BB 536 reduction throughout the refrigeration period in fermented goat milk supplemented with different fiber was dependent mainly on its source type. For instant, the highest total reduction was in fermented goat milk supplemented with barley bran; whereas, the maximum reductions in the first and second week refrigeration were in fermented goat milk supplemented with millet bran and sorghum bran, respectively. Hopefully, the final viable count of *strain BB536* in fermented goat milk after two weeks refrigeration storage was above the minimum number required to presence in probiotic to exert health benefits upon consumption, which was at least 6 log CFU/ml. Nevertheless, Kabeir *et al.,* (2005) reported that survivability of *B. longum BB536* under refrigeration storage of fermented Sudanese *Medida* (Sudanes cereal thin porridge) beverages was not affected for a period of 2 week. While Akalin *et al.,* (2004) noted a significant reduction on *B.longum BB46* in yogurt after 1 week refrigeration. Lactate and acetate accumulation caused limitation on growth and survival of *Bifidobacterium bifidum, Bifidobacterium breve*, and *Bifidobacterium longum* cultivated in milk (Desjardins *et al.,* 1990). This indicates that the viability of *Bifidobacterium* in fermented products was dependent on the carrier type and pH of the fermented products during the storage. Overall, most strains of *Bifidobacterium* are sensitive to pH values below 4.6. Therefore, for practical application, a pH value of the final product must be maintained above 4.6 to prevent the decline of *Bifidobacterium* populations (Tamime and Robinson, 1985; Modler *et al.,* 1990; Laroia and Martin, 1991). 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).

Reducing sugars during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Reducing sugars levels of the different fermented goat milk supplemented with inulin and different cereal bran (Sorghum, Barely and Millet) during refrigeration storage was presented in Table 2. The reductions in sugar were significant ($p<0.05$) in all fermented goat milk products (Table 2). The amount of sugar decreases in the first week refrigeration were 0.73,0.50,0.29 and 0.03%in fermented goat milk supplemented with barely bran,

sorghum bran, millet bran and inulin, respectively. On the contrary, reductions in the second week were not following similar trend of the first recording values of 0.28, 0.21, 0.19, and 0.11% in fermented goat milk supplemented with inulin, sorghum bran, millet bran and barely bran, respectively. These reduction rates are well correlation values with pH record present in table 3.

Table.1 The viable counts of *Bifidobacterium longum* BB536log (CFU/ ml) during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Type of supplementation	Storage period (weeks)		
	O (Initial)	One	Two
Inulin	7.83 ±0.16^{a}	6.98 ± 1.54	6.02 ± 0.08
Sorghum bran	8.43 ±0.03^{a}	7.03 ± 0.01^{b}	6.60 ± 0.49^{c}
Barley bran	7.25 ±0.06^{a}	6.49 ± 0.64^{b}	6.01 ± 0.04^{c}
Millet bran	7.96 ±0.02^{a}	6.48 ± 0.68^{b}	6.04 ± 0.15^{b}

* Values are mean ± SD for replicate independent runs.
**Values that bear different superscript letter in the same raw are significantly different at p<0.05.

Table.2 Reducing sugar during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Type of supplementation	Storage period (weeks)		
	O (Initial)	One	Two
Inulin	1.35 ± 0.01^{a}	1.32±0.01^{a}	1.04 ± 0.00^{b}
Sorghum bran	1.30 ± 0.01^{a}	0.80 ± 0.00^{b}	0.59 ± 0.00^{c}
Barley bran	1.31 ± 0.00^{a}	0.59 ± 0.01^{b}	0.48 ± 0.01^{b}
Millet bran	0.87 ± 0.00^{a}	0.59 ± 0.01^{b}	0.40 ± 0.00^{b}

* Values are mean ± SD for replicate independent runs.
**Values that bear different superscript letter in the same raw are significantly different at p<0.05.

Table.3 pH during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Type of supplementation	Storage period (weeks)		
	O (Initial)	One	Two
Inulin	5.11 ± 0.00^{a}	4.78± 0.01^{a}	3.81 ± 0.01^{b}
Sorghum bran	5.65 ± 0.01^{a}	4.27 ± 0.01^{b}	3.88 ± 0.01^{c}
Barley bran	4.40 ± 0.01^{a}	4.31 ± 0.01^{a}	3.69 ± 0.00^{b}
Millet bran	4.45 ± 0.03^{c}	3.97 ± 0.02^{b}	3.84 ± 0.01^{ab}

* Values are mean ± SD for replicate independent runs.
**Values that bear different superscript letter in the same raw are significantly different at p<0.05.

Table.4 TSS during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Type of supplementation	Storage period (weeks)		
	O (Initial)	One	Two
Inulin	13.23± 0.04[a]	13.25 ± 0.01[a]	11.61± 0.01[b]
Sorghum bran	11.70 ± 0.01[a]	10.33 ± 0.02[b]	11.94 ± 0.02[a]
Barley bran	13.56 ± 0.01[a]	14.09 ± 0.01[a]	12.51 ± 0.01[b]
Millet bran	14.21± 0.01[a]	14.44 ± 0.01[a]	12.62 ± 0.22[b]

*Values are mean ± SD for replicate independent runs.
**Values that bear different superscript letter in the same raw are significantly different at p<0.05

Table.5 Moisture during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Type of supplementation	Storage period (weeks)		
	O (Initial)	One	Two
Inulin	86.77± 0.04[b]	87.75 ± 0.01[a]	88.40 ± 0.07[a]
Sorghum bran	88.50 ± 0.28[a]	88.68 ± 0.02[a]	89.07 ± 0.02[a]
Barley bran	86.45 ± 0.71[a]	86.92 ± 0.07[a]	87.50± 0.01[a]
Millet bran	85.79 ± 0.01[b]	86.57 ± 0.07[a]	87.24 ± 0.00[a]

*Values are mean ± SD for replicate independent runs.
**Values that bear different superscript letter in the same raw are significantly different at p<0.05.

Table.6 Titratable acidity during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Type of supplementation	Storage period (weeks)		
	O (Initial)	One	Two
Inulin	0.65 ± 0.00[c]	0.69 ± 0.07[b]	0.96 ±0.01[a]
Sorghum bran	0.44 ± 0.07[c]	0.80± 0.00[b]	1.06± 0.00[a]
Barley bran	0.82 ± 0.00[c]	0.96± 0.00[b]	1.71± 0.07[a]
Millet bran	0.93 ± 0.00[d]	0.95 ± 0.00[d]	1.82± 0.71[a]

*Values are mean ± SD for replicate independent runs.
**Values that bear different superscript letter in the same raw are significantly different at p<0.05.

pH during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Referring to the result in Table 3 shows, the pH measurement of different fermented goat milk supplemented with inulin and cereal bran (Sorghum, Barely and Millet) during refrigeration storage. There was significant (p<0.05) reduction in pH due to supplementation with inulin and different cereal bran at two weeks of refrigeration (Table 3). The highest pH reduction in the first week was in fermented goat milk supplemented with sorghum bran (1.39pH), while the lowest reduction was in one supplemented with barely bran (0.09 pH). While the reductions recorded in the second

week of refrigeration were 0.98, 0.62, 0.38 and 0.13 in fermented goat milk supplemented with inulin, barely bran, sorghum bran and millet bran, respectively. In fact, reduction of pH is mainly due to the fermentation of sugars (Table 2) and accumulation of acid shown in Table 6. The created condition maintained a relatively acid pH even in large intestine, thus preventing the proliferation of pathogens causing unfavorable disorders. Nevertheless, it was reported that low pH and storage temperature are the most important determinations in *Bifidobacterium* mortality during storage (Sakai *et al.*, 1987). Shah *et al.*, (1995, 2000) also found similar decreases in pH values during storage of commercial yoghurts containing L. *acidophilus* and B. *bifidum*.

Changes in TSS during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

The effect of inulin and cereal bran (Sorghum, Barely and Millet) supplemented to fermented goat milk on TSS during refrigeration was obvious in Table 4**.** There were significant ($p<0.05$) increases in TSS of all types of fermented samples under refrigerated storage in the first week, then decreased in the second week; except for product supplemented with sorghum bran (Table 4). The amount of increases in the first week of refrigerated storage of different were 0.53, 0.23, and 0.0.02% in fermented goat milk with barely bran, millet bran and inulin, respectively. The increases could be attributed to the breakdown of macro-components to simple soluble ones. While in the second weeks the rate decreases were 1.58, 1.82, and 1.64% in fermented goat milk supplemented with barely bran, millet bran and inulin, respectively. The decreases might be due to slight fermentation of soluble sugars by strain BB536 together with the increases in moisture as a dilution factor (Table 5).

Changes in moisture during during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

By extend storage period moisture content of fermented goat milk supplemented with inulin and different cereal bran (Sorghum, Barely and Millet) was slightly increased (Table 5); due to the reduction in TSS by strain BB536 activity (Table 4). The slight increase in moisture might indicate slow enzymatic activity that break down the macro component into simple and releases of some water. Thus, over all levels of moisture after two weeks refrigeration storage of fermented samples increased as compared to their initial levels at the beginning of the storage (Table 5). This increase in moisture might indicate high enzymatic activity that break down the macro-component into simple and to the release of water.

Changes in titratable acidity during refrigeration storage of fermented goat milk supplemented with inulin and different cereal bran

Organic acids in fermented dairy products play an important role as natural preservatives and also contribute to the characteristic sensory properties of the product. As natural preservatives, they are known to inhibit certain pathogenic organisms, especially in yogurt (Fernandez-Garcia and McGregor, 1994). Table 6 shows the titratable acidity of different fermented goat milk supplemented with inulin and different cereal bran (Sorghum, Barely and Millet). Titratable acidity of the different fermented samples significant ($p<0.05$) increased by extended storage period for the two weeks correlating well with the reduction in pH (Table 3). The rates of titratable acidity increases in the first week refrigeration were 0.37, 0.14, 0.04 and 0.02% in fermented goat milk supplemented

with sorghum bran, barely bran, inulin and millet bran, respectively. In the second week the titratable acidity increases were even higher recording values of 0.88, 0.75, 0.28 and 0.25% in fermented goat milk supplemented with millet bran, barely bran, inulin and sorghum bran, respectively. Strain BB536 as well as other probiotic *Bifidobacterium* produces lactic acid, acetic acid, hydrogen peroxide, and bactericides are known to inhibit the development of pathogenic bacteria. It was also reported that lactic acid and acetic acid in fermented dairy product have antibacterial effect (Bullen et al,. 1976). The presence of organic acids in fermented dairy foods is due to several reasons, including bovine metabolic processes during the production of milk, bacterial growth, hydrolysis of milk fat or direct addition of acidulants. They are important indicators of bacterial metabolic activity in fermented dairy products like cheese and yogurt, and they also contribute to the taste and flavor of the product along with other volatile and semi-volatile compounds such as diacetyl and acetaldehyde (Marsili *et al.*, 1981; Panari, 1986; Bevilacqua and Califano, 1989; Monnet *et al.*, 1994).

In conclusion the sufficient numbers of viable strain *BB536* were maintained in different types of fermented goat milk supplemented with inulin and different cereal bran (sorghum, barely and millet) during refrigeration storage. These viable numbers of the strain after two weeks refrigeration fulfill probiotic food requirements. Therefore, this study can facilitate the development of cereal bran that exerts better viability maintenance as inulin even better during refrigeration of fermented goat milk.

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How to cite this article:

Limia Hashim Mohamed, Barka Mohammed Kabeir, Salma Elghali Mustafa, Salma Elzen Ibraheem and Saeed Abdullah Badahdah. 2020. Probiotic *Bifidobacterium longum* BB536 Viable Existence at Refrigeration Storage of Fermented Goat Milk Supplemented with Inulin and Different Cereal Bran (Sorghum, Barely and Millet). *Int.J.Curr.Microbiol.App.Sci.* 9(10): 2109-2118. doi: https://doi.org/10.20546/ijcmas.2020.910.257