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Research article

Probiotic Millet-Milk beverage Supplemented with Date Powder: A novel Functional Beverage

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Abstract

Bioavailability's of nutrients are the major concern rather than the supply of an adequate amount of nutrients in the diet. To increase mineral absorption, aqueous millet seed extract (MSE) and date powder were evaluated with or without milk and probiotics as functional beverages. Based on preliminary study, MSE with 7% date powder was prepared as a control beverage (C1). MSE in C1 was replaced by milk (1:1) to create C2. A part of C1 and C2 were inoculated with 1% probiotic bacteria, incubated at 37°C for 24 hto create probiotic T1, T2, beverages. Chemical, rheological, and sensory properties, as well as probiotic viability and biological activities were measured. Both C2 and T2 characterized by high solids, protein, carbohydrates, ash, and fibers, as well as exhibited a higher scavenging activity. During storage, T2 showed the highest viable count of probiotic bacteria (8.64 $\log_{10}$ cfu/mL), acidity (1.2%) and viscosity (140.6mPa.s.). Biologically, rats fed on C1 beverages had no significant effect on body weight gain, liver and kidney weight, and glucose content in plasma. However, the feeding on C2, T1 and T2 raised both calcium and iron content in plasma, as well as total antioxidant capacity and catalase increased, but malondialdehyde decreased. Both calcium and phosphorus concentrations in Tibia were the highest in rats fed on T1 and T2 beverages. The novel beverage can be used to produce a functional beverage, helps in the absorption of minerals, an increase bone density, and increases the antioxidant system in the body.

Introduction

Functional food is one of the areas that has received attention as there is still increasing recognition of the main role of food and beverages in disease prevention and treatment, so the production and consumption of functional foods have gained great importance [1,2]. Functional foods are defined as foods that have the characteristics of traditional food and have proven physiological benefits as well as reduce the risk of chronic diseases alongside basic food functions [3]. Functional beverages are considered the most active in functional foods because of their suitability and ability to meet consumer demands, and it is an excellent way to provide biologically active nutrients and compounds including probiotics and prebiotics [1].

Probiotics are defined as microorganisms when added in sufficient quantities that add beneficial health effects to the host [4]. The non-digestible food ingredients that selectively stimulate the growth or activity of probiotic bacteria in the large intestine and thus improve the human health condition are called prebiotic [5]. As for food products that combine a mixture of probiotics and prebiotic in a form of synergy,

they are called "Synebiotic" [6]. Numerous studies have shown the positive effects of probiotics, prebiotics, and synebiotic on calcium absorption and bone mineral density (BMD) or other parameters related to bone health [7].

As calcium is one of the essential micro-nutrients for bone health. It is a vital mineral in human metabolism as it accounts for about 1-2% of body weight in adults [8]. In the case of insufficient intake of calcium from food sources, the body will not be able to produce more of it and the persistent deficiency in the diet leads to a decrease in bone mass and increases the risk of developing osteoporosis [9]. Modern research is interested in developing alternative methods to prevent and treat calcium deficiency and osteoporosis and one of these potential methods is the consumption of probiotics. Studies have shown a positive effect of probiotics on enhancing the bioavailability of calcium and the consequent increase in bone mineral density, and most of the bacteria examined were *Lactobacillus* and *Bifidobacterium* [7, 10].

Millet is a functional food because it provides nutritional fibers and is rich in calcium, minerals, antioxidants and nutrients necessary for human health, and millet can also be used as fermented substrates for the growth of microorganisms (probiotics) which helps to increase the number of friendly bacteria that play a major role in promoting digestion [11]. Date powder can also be used in preparing functional foods, as a substitute for sugar in sweetening drinks and foods. It contains a high amount of iron and also contains fiber, proteins and fats [12]. Therefore, this work amid to evaluate physicochemical, microbiological and sensory properties as well as biological activities of aqueous millet seed extract and date powder in the presence or absence of milk and probiotics as functional beverages.

Materials and methods

Materials

Both pearl millet seeds (*Pennisetumglaucum*) and date powder (*Phoenix dactylifera L.*, dry and ground dates) were purchased from the local markets in the Qassim region, Kingdom of Saudi Arabia. The average components of date powder were 3.0, 69.0, 3.0 and 9.0% for proteins, carbohydrates, fat, and fiber, respectively. Fresh cow milk obtained from animal production farmer, faculty of Agriculture and Veterinary Medicine Qassim University, Kingdom of Saudi Arabia. The average components of fresh cow milk were 11.5, 3.1, 3.0, 4.7, and 0.7% for total solids, protein, fat, carbohydrates, and ash, respectively as well as 120 mg Ca/100 g milk. Strains of probiotic bacteria, *Lactobacillus acidophilus* and *Bifidobacteriumlongum*, were obtained from Christian Hansen, Denmark. De Man, Rogosa and Sharpe agar (MRS agar) was obtained from OXOID Ltd, Hampshire, UK. 1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma/Aldrich (St. Louis, MO., USA). All plasma analysis materials were obtained from Human Company, Germany.

Methods

Preparation of aqueous millet seed extract

The aqueous millet seeds extract (MSE) was prepared according to the method of Hassan *et al.* (2012), [13] with some modifications. Millet seeds washed, soaked in tap water at room temperature for 12 h to obtain the hydrated millet seeds, and then water filtered. Distilled water was added to the hydrated millet seeds at a ratio of 2:1, soaked at room temperature for 12 h, and boiled for 5 min. After cooling, the boiling water was centrifuged at 2500 rpm for 5 min, filtered, and stored at 5°C until used.

Preparation of probiotic cultures

B. longum and *L. acidophilus* were activated separately in pasteurized skim milk containing 0.5% yeast extract and incubated anaerobically at 37°C for 18 h, then stored at 5±2°C until used.

Functional fermented beverage making

Preliminary experiment and a sensory evaluation

The millet seeds extract (MSE) supplemented with 7% date powder to create MSE-date beverage (C1). The MSE was replaced by cow milk with a rate of 1:1 and 2: 1 and supplemented with 7% date powder to create MSE-milk beverage C2 and C3, respectively. All treatments were heated to 100ºC for 5 min, and then cooled to 37ºC. A part of the cold C1, C2, and C2 treatments were taken, inoculated with 1% mixed probiotic bacteria (1:1), and then incubated at 37°C for 18 h to create probiotic-MSE beverages T1, T2, and T3, respectively. All six resultant beverages were mixed thoroughly, dispensed in 200 ml glass bottlesand keep them in refrigerator at 5±2°C for 24 h. Thereafter, sensorial evaluation was achieved by 20 faculty members (Qaseem University, Kingdom of Saudi Arabia) to select the best one for judge's acceptability.Selected samplessubsequently stored at 5±2°C for 14 days and were analyzed in intervals for pH values, apparent viscosity, probiotic viability and sensory evaluation. Chemical composition, minerals and antioxidant activity were evaluated on day one at 5±2°C.

Chemical analysis

Moisture, ash, fat, total nitrogen and fiber were determined in fresh beverages according to Association of Official Analytical Chemists (AOAC) (2005) [14] **(**methods, 945.38, 923.03**,** 979.09 and 920.86**,** respectively). The protein content was obtained by multiplying the percentage of total nitrogen by 6.38 for milk, while by 5.7 for MSE. The total carbohydrates and solids were determined using the difference method according to the formula:

Soluble carbohydrates (%) = 100- (% moisture +% fat +% protein +% fiber +% ash).

Calibrated pH-meter (HANNA Instruments, pH 211, Italy), with a glass electrode, was used for directly measuring the pH value of beverages. Acidity content, expressed as percentage lactic acid, was determined by using 0.10 N NaOH to the phenolphthalein end point [15].

Apparent viscosity

The apparent viscosity of the functional millet beverages was measured using Visceter Fungi, lab Viscometer, Spain. The functional beverage, 400 mL, was poured into a cup of spindle R2 and was set at 100 rpm/ min for 2 min. The apparent viscosity was measured at 5±2°C, and recorded in triplicates in a mPa.s.

Antioxidant activity

The DPPH radical-scavenging activity of functional millet beverage samples was estimated using a stable DPPH radical (2, 2-diphenyl-1-picrylhydrazyl) assay as described by Brand-Williams *et al.,* (1995) [16]. The ability of the samples to scavenge free radicals (%) was then calculated using the following equation:

Radical scavenging activity (%) = $(A_{control} - A_{sample})/A_{control}$ x 100

Where: $A_{control}$ is absorbance of blank; A_{sample} is absorbance of sample.

Determination of minerals

The calcium, magnesium, phosphorus, and iron contents in the functional millet beverage samples were determined using Atomic Absorption Spectrophotometry (Shimadzu 6300 AAS AA/AE Spectrophotometer) according to the method of Bauer & Petrushevska (2000) [17].

The viability of probiotic bacteria

The viability of *L. acidophilus* and *B. longum* bacteria were estimated during cold storage periods at 5±2°C for 14 days using MRS medium according to Mortazavlan *et al*., (2007) [18]. The plates were incubated in anaerobic conditions for *B. longum* and in aerobic conditions for *L. acidophilus* at 37±2°C for 72 h. The numbers of bacteria were expressed as $\log_{10}$ cfu/mL.

Sensory evaluation

Sensory evaluation of the fermented functional millet beverages was performed during the storage periods at 5 ±2°C for 14 days by untrained judges (20). The samples were numbered randomly and submitted to the judges. Quality attributes were scored on a scale from 1 to 10, depending on the method American Public Health Association (APHA) (1992) [19]. The sensory evaluation included four criteria: excellent (10), very good (9-8), acceptable (7-6) and unacceptable or unpalatable (5-0), to determine five characteristics of the products: overall appearance, color, smell, taste and general acceptance.

Biological study

The animals in this research received care in compliance with the standard institution's criteria for the care and use of animals according to the procedures approved by the ethics committee of the National Research Centre (FWA 00014747).

Experimental design

Fifty adult male Swiss albino rats (*Musmusculus*), weighting 125-170 g were obtained from the King Saud University laboratory center, Riyadh, Saudi Arabia, and housed individually in plastic cages covered with metal mesh. The rats were fed on a standard diet contained of 20% crude protein, 4% crude fat, 3% crude fiber, 6% ash, 1% calcium, 0.6% phosphorus, 0.5% salt and 20, 20 and 2.2 IU/g vitamin A, B and E respectively, for 7 days prior to being used in the study, and maintained under controlled conditions of 24±2°C air temperature and a 12 h light-dark cycle. Rats were provided with water in clean glass bottles, and food water provided continuously and sufficiently. After adaptation period, rats were weighted and randomly divided into 5 groups and maintained for 6 weeks as following: GC-control group fed on standard diet only, GC1- fed on standard diet with C1 (positive control), GC2-fed on the standard diet with C2 (positive control2), GT1-: fed on standard diet with T1, and GT2- fed on standard diet with T2.At the end of the experiment, all rats were weighted and blood samples were collected from the retro-orbital venous plexus under anesthetized by the mixture of ethanol, chloroform, and diethyl ether (1:2:3) in heparinized tube. Heparinized tubes were centrifuged at 3000 rpm for 20 min. The plasma were separated at once by long pastier pipette and stored at -70°C, until biochemical measurements could be completed as soon as possible. At the end of blood collection, all animals were rapidly sacrificed and the liver and kidney of each animal were dissected, washed with saline, dried by placing them between two filter papers and then weighing directly. Also, the major leg bone (Tibia) was taken, cleaned of all soft tissues, recorded with wet weight and stored at -20°C.

Body weight gain and relative weight of the liver and kidneys

Body weight (g) of rats were recorded on day 1 (initial weight) after adaptation and on day 42 (end weight). Body gain of rats was calculated as follows:

Body weight gain (g) = End weight (day 42) - Initial weight (day 1The relative weight of the liver and kidneys was calculated from the following equation according to the standard methods of Association of Official Analytical Chemists (AOAC) (1990) [20] as follow:

Relative kidney weight = kidney weight in grams/body weight in grams x 100.

Relative liver weight = liver weight in grams/body weight in grams x 100.

Plasma biochemical analysis

Glucose in the blood plasma (mg/dL) was measured according to colorimetric method described by Trinder (1969) [21]. Calcium, magnesium, phosphorus and iron (mg/dL) were determined according to colorimetric methods as describe by Gitelman22. (1967) [22]; Grindler & Heth (1971) [23]; El-Merzabani*et al.* (1977) [24]; Williams *et al.* (1977) [25], respectively. The antioxidant capacity (TAC) of plasma was estimated (mmol/L) according to the method of Koracevic *et al.* (2001) [26]. Glutathione-S-Transferase (GST) was estimated (U/L) according to colorimetric method of Habig *et al.* (1974) [27]. Catalase (CAT) was measured (U/L) according tothe method described byAebi (1984) [28]. Malondialdehyde enzyme (MDA) of plasma was estimated (nanomol /mL) according to colorimetric method of Satoh (1978) [29].

Minerals content in Tibia

Tibia content of mineral elements (calcium-phosphorous-magnesium) was estimated according to Parvaneh *et al.* (2015) [30]. Tibia was dissected and cleaned of all soft tissue and the wet weight of the bone was recorded. The samples were dried and then transferred to the oven at 550°C for 24 h, and then ash was weighed. The ash samples were digested using HCL solution diluted with distilled water (3:1) and the concentration of the mineral elements (calcium-magnesium-phosphorus) was measured for each element separately by Atomic Absorption Spectrophotometry.

Statistical analysis

Statistical analysis [31] was performed using the GLM procedure with SAS software (2004). Duncan's multiple comparison procedure was used to compare the means. A probability of $P \leq 0.05$ was used to establish statistical significance.

Results and discussion

Sensory evaluation of the preliminary experimental beverages

Table 1 shows the sensory evaluation of functional fermented beverages to choose the best addition percentage of cow's milk to milt seed extract (MSE) with 7% dates powder and probiotic bacteria. There was no significant difference in sensory attributes ($p > 0.05$), including appearance and odor among all functional milt seed beverages. The taste, color, and overall acceptability scores were significantly higher ($P < 0.05$) in MSE-date beverage (C1) and probiotic MSE-milk (1:1) beverage (T2) than in MSE-milk (2:1) beverage (C3) and probiotic MSE-milk (2:1) beverage (T3). However, no significant differences were found between C1 and C2 or T1 and T2 even if the scores were numerically higher in C1 and T2 than in C2 and T1, respectively. Therefore the results obtained from sensory scores showed that the ratio of aqueous millet seed extract to milk, 1:1, was chosen as the suitable ratios to be used in the further study.

Properties of probiotic millet-milk beverage supplemented with date powder

Based on the results of the initial sensory evaluation of the probiotic millet-milk beverage supplemented with date powder, the best treatments (C1, C2, T1, and T2) selected to study their physicochemical, and microbiological properties as well as study some of their biological activities.

Chemical properties

The average components of aqueous millet seeds extract (MSE) were 98.77, 0.6, 0.02, 0.6 and 0.01% for moisture, protein, fat, carbohydrates, and ash, respectively.

The total solids, fat, protein, non-fat solids, carbohydrates, fibers, and ash contents of probiotic beverages T1 and T2 as compared to control beverage C1 and C2 are presented in Table 2. The composition content varied significantly ($P < 0.05$) between the types of beverage. The content of total solids, fat, protein, non-fat solids, and carbohydrates was higher in C2 and T2 than in C1 and T1, due to the high protein, fat and carbohydrates in milk compared to millet seed extract [13, 32]. However, no mush differences were observed in fibers and ash content among all beverage treatments.

Minerals content

The minerals content of probiotic beverages T1 and T2 as compared to control beverage C1 and C2 are given in Table 3. In general, functional millet-date beverage characterized with high minerals content.This may be due to the high content of millet seeds from the minerals which facilitate the soaking process from being transferred to the extract [33].

Dates also contain essential minerals; for example, 100 g dates contain 696 mg K, 90 mg Fe, 362 g Cu, and 90 mg Mg [34]. Replacing MSE by milk had significant effect ($P \leq 0.05$) on Ca and P. This means that the cow milk content is differentto that of millet seed extract. However, the addition of milk can improve Ca, Mg and P content. The Ca content increased from 47.2±0.87 and 46.9±1.16 in C1and T1 to 480.1±6.10 and 472.1±9.02mg/100g in C2 and T2, while P content increased from 32.6±2.16 and 35.8±2.15in C1and T1 to 316.7±19.47 and 338.2±8.10mg/100g in C2 and T2.The increase in these minerals is due to milk, as milk is rich in calcium and phosphorous [35]. Whereas, the iron content in C1 and T1 were higher if compared to C2 and T2. Millet and dates are more abundant in iron and magnesium than milk. The soaking process improves the availability of minerals in MSE [33].

Table 1. Sensory evaluation of preliminary experimental for MSE-date beverage with or without milk and probiotic.

Treatments	Appearance	Odor	Taste	Color	Overall acceptance
C1	8.95^{a}±0.23	8.65^{a}±0.22	8.80^{a}±0.21	9.40 ^{a}±0.16	9.05^{a}±0.22
C2	8.55^{a}0.28	8.85^{a}±0.27	8.35^{ab}±0.31	8.90^{ab}±0.22	8.60^{ab}±0.32
C3	8.80 ^{a}±0.15	8.70^{a}±0.30	7.95 ^{b}±0.18	8.30^{b}±0.26	8.15^{bc}±0.18
T1	8.25^{a}±0.36	8.20 ^{a}±0.37	8.10^{ab} ±0.34	8.70 ^{b}±0.30	8.25^{bc}±0.26
T2	8.95^{a}±0.23	8.65^{a}±0.22	8.80^{a}±0.21	9.40^{a}±0.16	9.05^{a}±0.22
T3	8.15^{a}±0.30	8.45 ^{a}±0.28	7.70 ^{b}±0.24	8.30^{b}±0.20	7.80 ^{c}±0.26

Means (n-3, ±SE) with the same letters are no significantly different ($P < 0.05$); C1, millet seed extract (MSE) with 7% date powder; C2, MSE and milk (1:1) with 7% date powder; C3, MSE and milk (2:1) with 7% date powder; T1, MSE with 7% date powder and probiotic bacteria; T2, MSE and milk (1:1) with 7% date powder and probiotic bacteria; T3, MSE and milk (2:1) with 7% date powder, and probiotic bacteria.

Table 2. Chemical properties of probiotic millet-milk beverage supplemented with date powder.

Treatments	Fats (%)	Protein (%)	Total solids (%)	Soluble carbohydrates (%)	Fibers (%)	Ash (%)
C1	0.06^{b} ±0.01	2.43^{b} ±0.11	7.1^{b} ±0.02	4.21^{b} ±0.13	0.10^{a}±0.02	0.30^{b} ±0.003
C2	1. 65^{a} ±0.06	3.65^{a} ±0.13	11.88^{a} ±0.17	5.87^{a} ±0.23	0.14^{a} ±0.003	0.57^{a} ±0.004
T1	0.24^{b} ±0.05	2.72^{b} ±0.05	7.60^{b} ±0.63	4.23^{b} ±0.08	0.1 ^{a} ±0.007	0.30 ^{b} ±0.003
T2	1.55^{a} ±0.01	4. 03^{a} ±0.09	12.03^{a} ±0.07	5.75^{a} ±0.08	0 .12^{a} ±0.009	0.56 ^{a} ±0.004

Means (n-3, ±SE) with the same letters are no significantly different ($P < 0.05$); C1: aqueous millet seed extract (MSE) with 7% date powder; C2, MSE and milk (1:1) with 7% date powder; T1: C1+ probiotic bacteria; T2: C2 + probiotic bacteria.

Table 3. Minerals contentof probiotic millet-milk beverage supplemented with date powder.

Treatment	Calcium (mg/100 g)	Magnesium (mg/100 g)	Phosphorus (mg/100 g)	Iron (mg/100 g)
C1	47.2 ^{b}±0.87	47.2 ^{b}±0.87	32.6 ^{b}±2.16	1.99 ^{a}±0.06
C2	480.1 ^{a}±6.10	59.0 ^{a}±0.97	316.7 ^{a}±19.47	1.62 ^{b}±0.023
T1	46.9 ^{b}±1.16	46.9 ^{b}±1.16	35.8 ^{b}±2.15	2.12 ^{a}±0.09
T2	472.1 ^{a}±9.02	60.0 ^{a}±0.25	338.2 ^{a}±8.10	1.65 ^{b}±0.10

Means (n-3, ±SE) with the same letters are no significantly different ($P < 0.05$); C1: aqueous millet seed extract (MSE) with 7% date powder; C2, MSE and milk (1:1) with 7% date powder; T1: C1+ probiotic bacteria; T2: C2 + probiotic bacteria.

Antioxidant activity

Antioxidant activity of MSE-date beverage with or without milk and probiotic, measured as DPPH radical scavenging is shown in Figure 1. The MSE-date beverage (C1) showed anantioxidant activity against the DPPH radicals. Millet is rich in antioxidant compound such as, phenols and Tannins that can contribute to the important antioxidant activity of health, aging and metabolic syndrome [36]. Date powder is also a good source of a mixture of antioxidants including ascorbic acid, carotenoids, flavonoids and polyphenols [37]. Replacing the MSE with cow milk (1:1, C2) improved the antioxidant activity compared with C1.The addition of probiotic bacteria also increased the antioxidant activity of millet-milk beverage T2 compared toT1 and C2. The fermentation process may increase the protein hydrolysis and produce many of bioactive peptides which act as antioxidant compounds. Similarly, the antioxidant activity significantly increased ($p < 0.05$) as cheese proteolysis increase [38]. Shazly *et al.* (2019) [39] found that the high antioxidant capacity of casein was related to the degree of hydrolysis. Additionally, Đorđević *et al.* (2010) [40] reported that fermented grains (buckwheat, wheat germ, barley and rye) with *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* exhibit high antioxidant activities. Virtanen *et al.* (2007) [41] found that fermentation of milk with certain strains of lactic acid bacteria increased antioxidant activity. The production of peptides from milk fermentation has a positive effect on human health, specifically the anti-mutagenic and antioxidant properties [42].

Viable counts of probiotic during storage

Figure 2a and b show the viability of probiotic bacteria in probiotic-MSE beverage samples, T1 and T2, during storage periods at 5±2°C for 14 days. In general, the counts of *B. longum* and *L. acidophilus* were higher and more stable in millet-milk beverage (T2) than in millet

beverage without milk (T1), during storage periods. The *B. longum* counts of T1 showed the highest viability on day 1 (7.2 $\log_{10}$ cfu/mL), but the counts fluctuated until day 10 and then cannot be detected at the end of storage (Figure 2a). On the contrary, the *B. longum* counts of T2 showed a gradual increase until day 5 (7.1 $\log_{10}$ cfu/mL) and then the counts were more stable or slight decrease until day 14 (6.8 $\log_{10}$ cfu/mL). The *L. acidophilus* counts showed close viability in both T1 and T2 on the first day (8.4 and 8.64 $\log_{10}$cfu/mL, respectively), and decreased thereafter; the decrease was more pronounced in T1 (Figure 2b). This means that milk may improve the survival of probiotic bacteria permanently. A similar observation was found by Di Stefano *et al.* (2017) [43], who reported that the numbers of *L. rhamnosus* GR-1 bacteria and *Str. thermophilus* C106 were better grown in fermented products containing millet and milk. When comparing *B. longum* and *L. acidophilus, L. acidophilus* grew better and more stable (in required limit) during storage than *B. longum* in both T1 and T2 (Figure 3a and b). This can be explained by the more sensitivity of the *B. longum* than *L. acidophilus* by increasing the acidity. Almeida *et al.* (2008) [44], reported that the sensitivity of the probiotic culture is affected by low pH values and is dependent on the strains. However, the presence of milk components in the treatment T2 may work to protect and enhance the presence of *B. longum* compared to its vitality and presence in the treatment T1.

Probiotic bacteria help restore the presence of natural flora of microbes in the colon when bacteria levels decrease through antibiotics, chemotherapy, or disease. The Food and Agriculture Organization and World Health Organization [45] report that probiotics are living organisms that live when given in sufficient quantities (10^6 to 10^7cfu/ml) health benefit to the host [46, 47]. The *L. acidophilus* and *Bifidobacteria* spp were able to digest 60-80% of parts of pearl millet fibers[48]. Date powder is rich in sugar, protein, dietary fiber, minerals and some vitamins [49-51]. About 85% of the total carbohydrates in dates are in the form of simple sugars like glucose and fructose [34]. Glutamic, aspartic, glycine, proline and leucine are among the amino acids prevalent in dried dates. All these compounds help to enhance the growth of probiotic bacteria in the functional drink and make them in the required numbers until the end of the storage period.

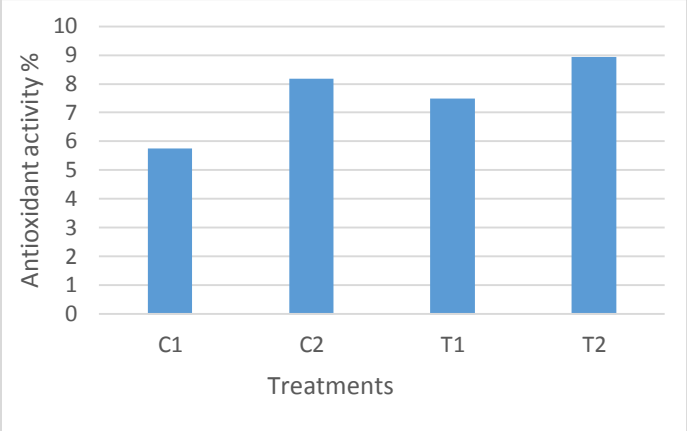

Figure 1. DPPH radical's scavenging activity of probiotic millet-milk beverage supplemented with date powder. Means (n-3, ±SE) with the same letters are no significantly different (P < 0.05); C1: aqueous millet seed extract (MSE) with 7% date powder; C2, MSE and milk (1:1) with 7% date powder; T1: C1+ probiotic bacteria; T2: C2 + probiotic bacteria.

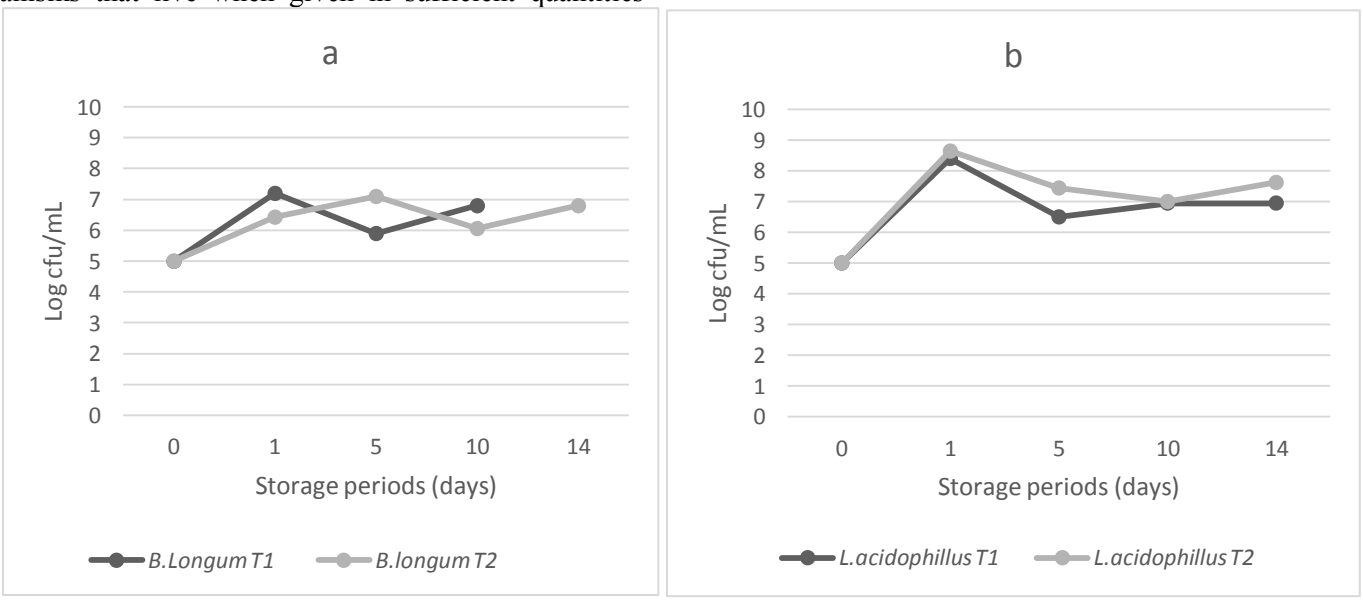

Figure 2a and b. The viability of probiotic bacteria, (a) *B. longum* and (b) *L. acidophilus* in MSE-date beverages with or without milk during storage at 5±2°C for 14 days. T1: MSE with 7% date powder and probiotic bacteria; T2: MSE and milk (1:1) with 7% date powder and probiotic bacteria.

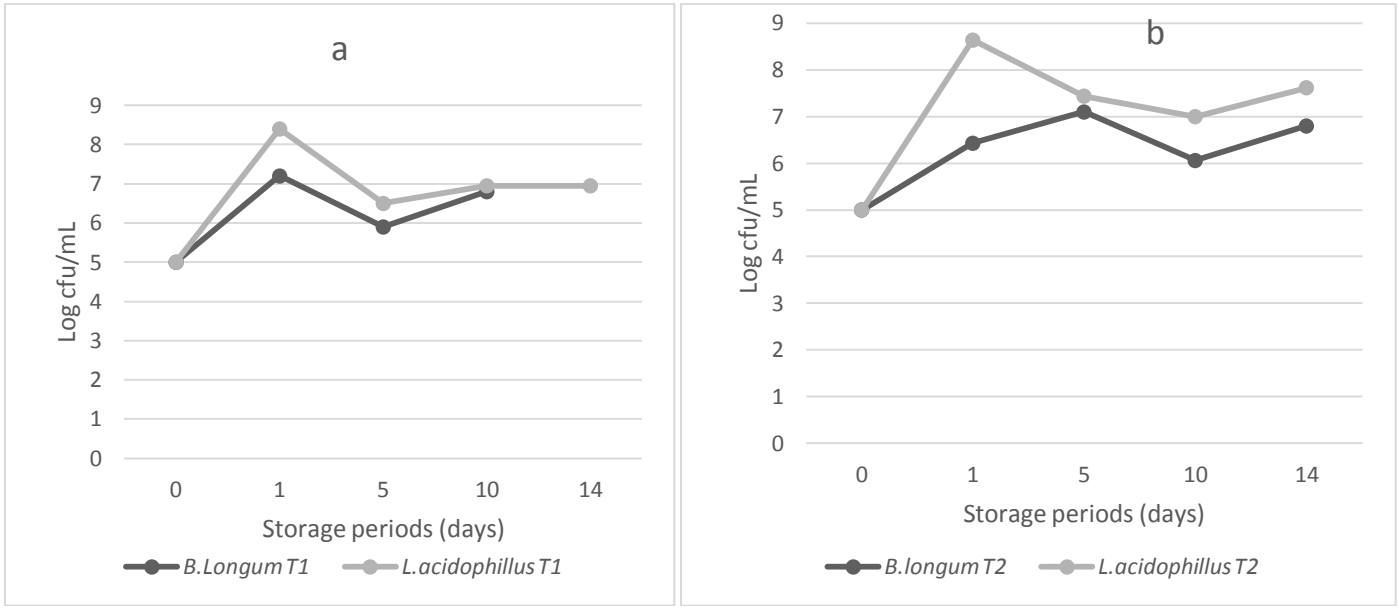

Figure 3a and b. The comparison of the viability of probiotic bacteria, (a) *B. longum* and *L. acidophilus* in MSE-date beverages without milk and (b) *B. longum* and *L. acidophilus* in MSE-date beverages with milk probioticbacteria during storage at 5±2ºC for 14 days. T1: MSE with 7% date powder and probiotic bacteria; T2: MSE and milk (1:1) with 7% date powder and probiotic bacteria.

Change in pH value and acidity content value

Figure 4a and b illustrate the pH value and acidity content of fermented millet beverage during storage periods. In general, probiotic millet-date beverage (T2) was characterized by high acidity and low pH compared to T1. This result was agreed with Helland *et al.* (2004) [52], who stated that the quantity of lactic acid produced by lactic acid bacteria from cereals with milk was the highest if compared with cereals without milk. The decrease in pH or the increased acidity content was due to the activity of probiotic bacteria and their fermentation of sugars and the production of lactic acid. The beverage contains many factors that encourage the growth of probiotic bacteria. The presence of milk lactose in T2 led to significant decrease of pH and increased ($P < 0.05$) acidity content, respectively, due to the preference of probiotic bacteria for using lactose sugar [53, 54]. During storage, a drop in the pH, acidity increased, ($P < 0.05$) of the probiotic millet-date beverage observed on day 1, and then the changes were not significant ($P > 0.05$) until day 14.

Change in apparent viscosity

As shown in Fig 5, the apparent viscosity of T2 was higher significantly than that of T1 during different storage periods ($P < 0.05$). The higher viscosity of T2 sample may be due to the increase in the total solids content, and the coagulation of milk by probiotic bacteria compared with T1 sample [55]. However, apparent viscosity of T2 sharply and gradually decreased as storage period increased ($P < 0.05$). A similar, but less marked, slight was observed in apparent viscosity of T1. Similar observation was found by El-Shenawy*et al.* (2019) [56], in a probiotic beverage made from tiger-nut extract and milk permeates. The probiotic bacteria have the ability to produce amylase enzyme, which hydrolyze the starch chains and decrease the viscosity. Additionally, the decrease of viscosity may be also due to the decrease in the number of probiotic bacteria compared to their numbers in fresh samples. Lactic acid bacteria are neutrophilic, that have optimum pH growth. This could be explaining why the probiotic numbers declined with the reduction in pH during storage periods [57]. Another explanation can be made, the lower viscosity can also be attributed to the reduced ability of the protein to bind to water as a result of a slightly higher pH value during storage and a decrease in lactic acid production resulting from a decrease in the numbers of probiotic bacteria [58].

Sensory evaluation

Functional MSE beverage, T1 and T2, were sensory evaluated as a fresh and during the storage period at 5±2°C for 14 days (Table 4). The evaluation was made on the basis of the general appearance, color, odor and general acceptance. On day 1, there were no significant differences ($P > 0.05$) in the appearance, color, odor, taste, and general acceptance between T1 and T2. However, the T2 had a higher taste and overall acceptance scores ($P < 0.05$) than T1 on day 14, while no difference was found in appearance and odor between them. In general, storage period decreased the color and taste scores of T1, while decreased only the color score of T2.However, odor, taste and overall acceptance remained without decrease during storage periods. The components of fermented functional beverage, which is included

millet extract with or without milk, and the presence of 7% dates powder gave a delicious taste and aroma that tends to the sweet and sour taste, due to the fermentation of probiotic bacteria for sugars and the production of a desired smell and taste, which increased the degree of acceptance of the arbitrators for the beverage. Di Stefano *et al.* (2017) [43], who reported that fermentation of millet with probiotics produced fresh and delicious fermented foods as the results of the sensory evaluation indicated a preference for foods fermented millet with milk. It is also consistent with the study of Fasreen *et al.* (2017) [59], who developed a probiotic beverage consisting of the health benefits of millet and probiotics. The acidity of the product has also an indirect positive effect on sensory properties such as the final taste and aroma [60].

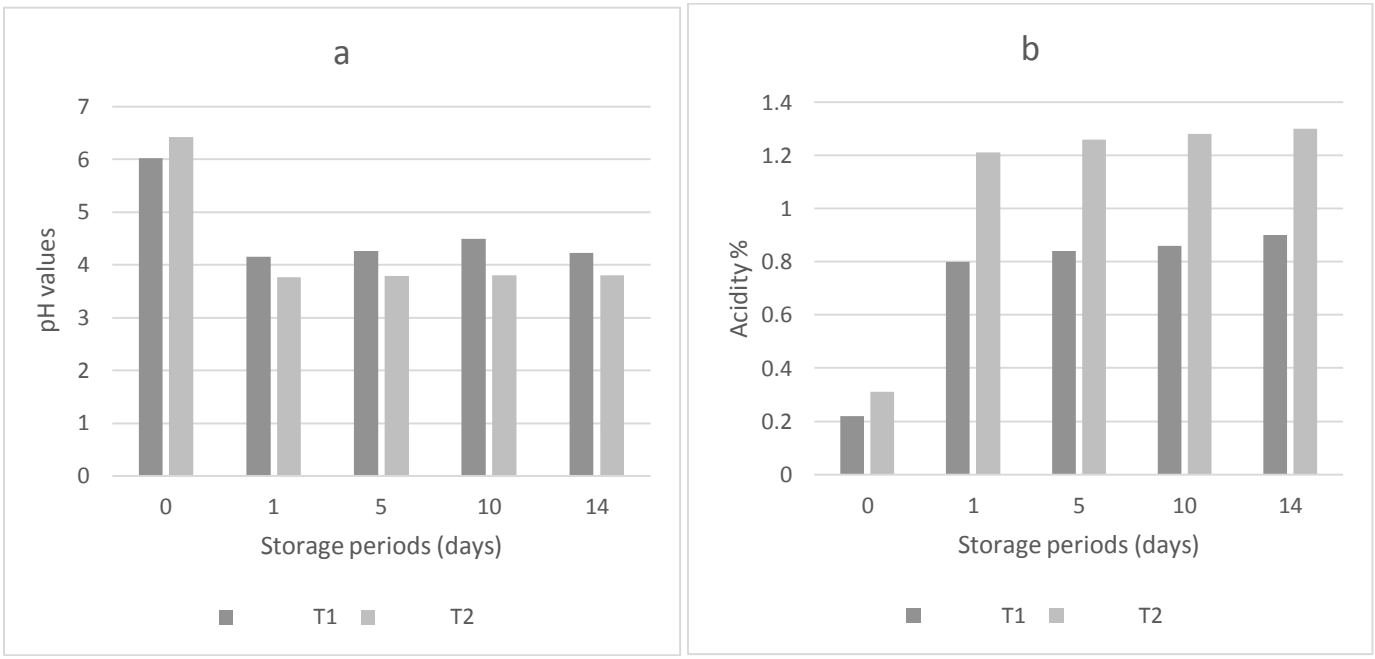

Figure 4a and b. The pH value (a) and acidity content (b) of MSE-date beverage with or without milk and probiotic bacteria during at 5±2°C for 14 days. T1: MSE with 7% date powder and probiotic bacteria; T2: MSE and milk (1:1) with 7% date powder and probiotic bacteria.

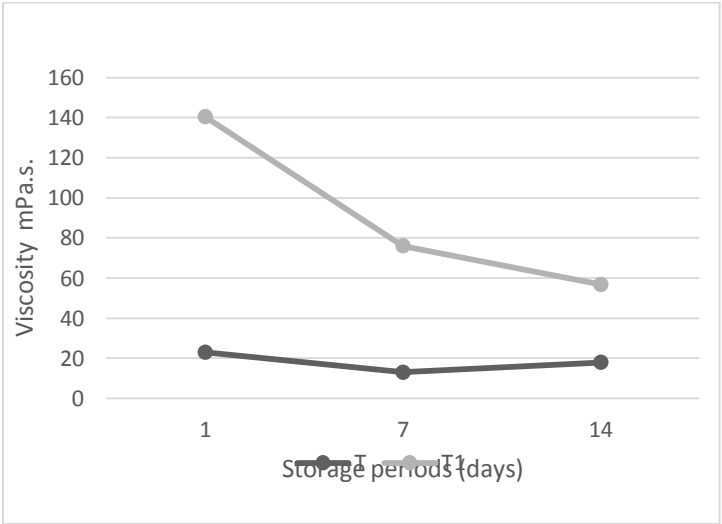

Figure 5. Apparent viscosity of MSE-date beverages with or without milk and probiotic bacteria during storage at 5±2ºC for 14 days. T1: MSE with 7% date powder and probiotic bacteria; T2: MSE and milk (1:1) with 7% date powder and probiotic bacteria.

Table 4. Sensory evaluation of probiotic millet-milk beverage supplemented with date powder during storage at 5±2ºC for 14 days.

Beverages	Storage period days	Appearance	Odor	Taste	Color	Overall acceptance
T1	Fresh	8.25^{a}±0.36	8.20^{b}±0.37	8.10^{ab}±0.35	8.70^{ab}±0.30	8.25^{b}±0.27
	7	8.25^{a}±0.28	7.90^{ab}±0.39	8.65^{a}±0.28	7.70^{b}±0.35	8.65^{ab}±0.33
	14	8.20^{a}±0.33	9.05^{a}±0.16	7.75^{b}±0.29	8.10^{b}±0.38	8.25^{b}±0.29
T2	Fresh	8.95^{a}±0.23	8.65^{ab}±0.23	8.80^{a}±0.21	9.40^{a}±0.17	9.05^{a}±0.22
	7	8.10^{a}±0.26	8.20^{b}±0.25	8.90^{a}±0.28	7.80^{b}±0.24	9.20^{a}±0.25
	14	8.32^{a}±0.25	8.80^{a}±0.15	8.65^{a}±0.22	7.75^{b}±0.46	8.90^{ab}±0.12

Means (n-3, ±SE) with the same letters are no significantly different (P < 0.05); T1: MSE with 7% date powder and probiotic bacteria; T2: MSE and milk (1:1) with 7% date powder and probiotic bacteria.

Biological activities of probiotic millet-milk beverage supplemented with date powder.

Body weight gain and the rate of increase in liver and kidney weight

Body weight gain (g) and increasing rate (%) of normal rat groups fed on standard diet only (GC), standard diet with C1 (GC1), standard diet with C2 (GC2), standard diet with T1 (GT1) and standard diet with T2 (GT2) for 42 days are shown in Table 5. Compared to standard diet, the body weight gain was slightly increased in GT1, while slightly decreased in GC1, GC2 and GT2; the difference was not significant. The reduction in body weight gain of groups treated with MSE beverages may be due to their bioactive constituents such as phenols and flavonoids which act as lipase inhibitor as well as its ability to bind cholesterol and bile acids, and increase their removal via the feces [61, 62]. Similar, no significant difference (P > 0.05) was observed in increasing rate of liver and kidney weight among all rat groups. These results were agreed with Hunt *et al.* (2008) [63] who found that there was no effect on food intake and weight gain and calcium concentrations in the kidneys and muscles of Sprague-Dawley female rats after 13 weeks of consuming of modified diet (AIN-93G) containing 1 to 7 g of Ca/Kg.

Biochemical changes

Plasma glucose and minerals

As shown in Table (6), all rat groups fed on standard diet with all MSE beverages had no significant on plasma glucose compared to control group (GC). The plasma glucose content ranged between 120.9 to128.3 mg/dLin all rat groups. Also, Table 6 shows that both Mg and P content of plasma had no affected by the MSE-date supplemented with milk or/and probiotic bacteria. On the contrary, feeding rats on a standard diet and MSE-date supplemented with milk or/and probiotic bacteria (GC2, GT1, and GT2) resulted in a slight increase in the plasma Ca content (P > 0.05), but a significant increase in the plasma Fe content (P < 0.05) if compared to GC and GC1. Calcium is easily ionized in fermented milk due to low pH, which in turn improves intestinal calcium absorption [64].The reason for the increased concentration of magnesium in the groups GT2 and GT1can also be attributed to feeding these groups on beverages that contain high magnesium concentrations compared to other beverages, (see Table No 3).The prebiotics in SME beverages stimulate the growth of probiotics in the intestine and enhance the transport of Ca through the intestinal barrier. Bifidobacterium is also producing short-chain fatty acids (SCFA) that can lower the acidity of the intestine, thus increasing bioavailability and absorption of minerals such as Ca and Fe [65-67]. Gohel *et al.* (2016) [68], showed that the feeding on fermented milk using *L. helveticus* MTCC 5463, in the elderly for 3 years, increases the Ca level in the blood serum. Millet is a good source of nutritional minerals such as Mn, Fe, and P which play a major role in energy synthesis in the body [11, 69].

Total antioxidant capacity and glutathione-S-transferase catalases and lipid peroxide

The plasma of rats fed on a standard diet and MSE-date beverage supplemented with or without milk and probiotic exhibited showed high total antioxidant capacity (TAC); however, the difference was only significant (P < 0.05) in GT1 and GT2 when compared to GC (Table 7).This result could be attributed to the milk fermentation with lactic acid bacteria produce some biologically active compounds that have a positive impact on human health, specifically anti-mutagenic and antioxidant properties [42]. Similarly, the feeding rats on a standard diet and MSE-date beverage supplemented with or without milk and probiotic increased the concentrations of catalase in plasma of rats (P < 0.05). The catalase concentration was the highest in GC2 (848.9±27.1 IU/L), followed by both GT1 and GT2, (825±10.4 and 824±8.07 IU/L) and then GC1 (347.4±41.4 IU/L). On the contrary, the malondialdehyde concentrations decreased in plasma of rats treated with probiotic millet-milk beverage supplemented with date powder; the decrease was significant only in plasma of GT1 and GT2. A negative

correlation was found between catalase and malondialdehyde concentrations. A similar observation was found by Al-Humaid *et al.* (2010) [70] in date- camel milk mixture, as a preventive meal against oxidative stress of cells in experimental rats. The phytochemical analysis of dates showed that they contain phenols, carotenoids, flavonoids, which exhibit strong antioxidant, anticancer, and antiviral agents [71]. The soluble and insoluble phenolic extracts of many whole grains in millet types (Kudu, finger, foxtail, brusu, pearl and little millet) are rich sources of phenolic compounds and show antioxidant activity [72]. Milk also contains many antioxidants, the most important of which are mineral selenium and vitamins E and A [32]. However, there was no significant difference was found in plasma glutathione-S-transferase among all rats groups.

Mineral concentrations in Tibia of rats.

Table 8 shows the minerals content of Tibia of rats treated with probiotic millet-milk beverage supplemented with date powder. In general, supplementation of MSE-date beverage with milk and probiotic in separately or in combination increased Ca content of Tibia of rats. The Ca content increased from 182.7±8.95(mg/g) in GC to 188.1±7.70, 224.4±10.93and 225.6±18.54(mg/g) in GC2, GT1 and GT2 respectively. The increase in Ca content in Tibia may due to the presence of probiotics in the GT1 and GT2 as well as the presence of prebiotics. Prebiotics and probiotic bacteria improve the production of short chain fatty acids (SCFA) which also improve the absorption of mineral elements such as calcium and magnesium. Moreover, prebiotic fermentation in the intestine can increase SCFA production which lowering the pH in the intestine, and thus can increase the absorption capacity of calcium needed for bone formation [66, 73, 74]. However, there was no significant difference ($P > 0.05$) in the concentration of Mg in the Tibia of all treated groups compared to the control group. Slight increase was found in P content of Tibia of groups GT1 and GT2 compared to control group; the difference was not significant among all groups ($P > 0.05$).

Table 5. Body weight gain and increase rate in liver and kidney weight of rats treated withprobiotic millet-milk beverage supplemented with date powder compared to standard diet.

Groups	**Body weight gain (g)**	**Increasing rate (%)**		
		Body weight	Liver weight	Kidney weight
GC	129.6^{a} ±11.11	46.3^{a} ±2.26	0.74 ^{a}±0.02	3.45 ^{b}±0.08
GC1	115.9 ^{a}±7.39	43.8^{a}±2.04	0.76 ^{a}±0.02	3.84 ^{a}±0.14
GC2	125.1 ^{a}±5.69	45.9^{a} ±1.53	0.74 ^{a}±0.04	3.42 ^{b}±0.06
GT1	136.6 ^{a}±7.91	47.9^{a} ±1.78	0.74^{a}±0.01	3.52 ^{b}±0.05
GT2	123.7 ^{a}±8.57	45.4^{a}±2.03	0.69 ^{a}±0.01	3.28 ^{b}±0.04

Means (n-10, ±SE) with the same letters are no significantly different ($P < 0.05$); GC: control group fed on standard diet only; GC1: fed on standard diet with C1; GC2: fed on the standard diet with C2; GT1: fed on standard diet with T1; GT2: fed on standard diet with T2.

Table 6. Plasma glucose and mineral content of rats treated withprobiotic millet-milk beverage supplemented with date powder compared to standard diet.

Groups	**Glucose (mg/dL)**	**Calcium (mg/dL)**	**Magnesium (mg/dL)**	**Iron (mg/dL)**	**Phosphorus (mg/dL)**
GC	120.9^{a}±6.5	10.92^{b}±0.07	1.48^{b}±0.00	85.1^{b}±0.54	9.07^{a}±0.40
GC1	128.3^{a}±4.51	11.08^{b}±0.08	1.47^{bc}±0.00	86.3^{b}±0.43	8.72^{a}±0.15
GC2	121.5^{a}±3.83	11.63^{a}±0.17	1.46^{c} ±0.00	101.4^{a}±3.21	9.10^{a}±0.42
GT1	123.4^{a}±4.36	11.72^{a}±0.15	1.53^{a}±0.00	97.2^{a}±3.79	9.43^{a}±0.15
GT2	120.9^{a}±6.56	11.84^{a}±0.17	1.55^{a}±0.00	100.0^{a}±1.70	9.07^{a}±0.40

Means (n-10, ±SE) with the same letters are no significantly different ($P < 0.05$); GC: control group fed on standard diet only; GC1: fed on standard diet with C1; GC2: fed on the standard diet with C2; GT1: fed on standard diet with T1; GT2: fed on standard diet with T2.

Table 7. Total antioxidant capacity and glutathione-S-transferase, catalases and lipid peroxide in plasma of rats treated withprobiotic millet-milk beverage supplemented with date powder compared to standard diet.

Groups	TAC (mmol/L)	GST (IU/L)	CAT (IU/L)	MDA (µmol/L)
GC	1.28 ^{c}±0.09	467.3^{a}±39.1	347.4 ^{c}±41.4	7.02 ^{a}±0.55
GC1	1.46^{abc}±0.04	438.9 ^{a}±25.9	460.9 ^{b}±27.1	6.57 ^{ab}±0.45
GC2	1.36 ^{bc}±0.10	446.5 ^{a}±39.2	848.9 ^{a}±7.52	6.08 ^{abc}±0.37
GT1	1.53 ^{ab}±0.09	492.1 ^{a}±31.5	825.9 ^{a}±10.4	5.06 ^{c}±0.63
GT2	1.63 ^{a}±0.03	497.3 ^{a}±28.2	824.0 ^{a}±8.07	5.29^{bc} ±0.12

Means (n-10, ±SE) with the same letters are no significantly different (P < 0.05); GC: control group fed on standard diet only; GC1: fed on standard diet with C1; GC2: fed on the standard diet with C2; GT1: fed on standard diet with T1; GT2: fed on standard diet with T2; TAC: total antioxidant capacity; GSA: glutathione-S-transferase; CAT: catalase; MDA: malondialdehyde.

Table 8. Minerals content in Tibia of rats treated withprobiotic millet-milk beverage supplemented with date powder compared to standard diet.

Groups	Calcium (mg/g)	Magnesium (mg/g)	Phosphorous (mg/g)
GC	182.7^{b}±8.95	2.28^{a}± 0.14	83.4^{b} ±3.59
GC1	183.2^{b}±7.14	2.25^{a} ± 0.02	83.7^{b} ±0.48
GC2	188.1^{ab} ±7.70	2.03^{a}± 0.11	88.0^{ab} ± 3.03
GT1	224.4^{a} ± 10.93	2.36^{a}± 0.17	98.2^{a} ± 5.22
GT2	225.6^{a} ± 18.54	2.16^{a}± 0.15	98.7^{a} ±4.63

Means (n-10, ±SE) with the same letters are no significantly different (P < 0.05); GC: control group fed on standard diet only; GC1: fed on standard diet with C1; GC2: fed on the standard diet with C2; GT1: fed on standard diet with T1; GT2: fed on standard diet with T2

Conclusion

This study elucidated that the MSE-date beverage with or without milk provide the required numbers of *B. longum* and *L. acidophilus* with increasing in the vitality of bacteria by milk addition. Moreover, biological activities of MSE-date beverage with or without milk showed increasing of Ca and P content of Tibia and high total antioxidant in plasma of rats. The study concluded that the MSE-date beverage contained milk gave a delicious taste and aroma that tends to the sweet and sour taste, which increased the degree of acceptance of the judges for the beverage.

Conflicts of Interest

The authors declare no conflict of interest.

Author contributions

All the authors have contributed equally in designing, drafting the manuscript as per the journal submission format. All authors read and approved the final manuscript.

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