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DEVELOPMENT OF ANTIOXIDANT RICH PROBIOTIC DRINK: A BOOTSTEP APPROACH IN NUTRACEUTICAL

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ABSTRACT
Probiotic formulation beckons the milk nutraceutical industries nowadays and India being the largest producerof milk has a vast scope in nutraceutical market. The objectives of the present study were to determine the effect of medically important herbs *Tulsi* or *Kalmegh* on yogurt fermentation, probiotic bacterial count and antioxidant activity of the fermented yoghurt during thestorage period of 21 days. *Tulsi* or *Kalmegh* water extact was mixed with milk (6%v/v) and the mixture was fermented with probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacterium bifidum* to form herbal-yogurt. Changes in pH, total titratable acids and syneresis were monitored and the viability of probiotic bacteria was evaluated during refrigerated storage. Antioxidant activity was measured by 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) assay. The addition of herbs significantly increased the antioxidant activity of yogurts compared to plain-yogurt at all storage periods.The highest antioxidant activity was recorded for *Tulsi*-yogurt followed by *Kalmegh* -yogurt and plain-yogurt. The products thus developed have potential use as functional food enriched with antioxidants for consumers in international market.

KEYWORDS: Probiotics, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, herbal-yogur, antioxidant activity.

INTRODUCTION
Probiotic bacteria are defined as "living micro-organisms", which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" (Guarner and Schaafsma, 1998). Two such species include *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. They are pH and bile resistant, survive passage through and colonize in the gastrointestinal tract. They exhibit antagonistic effects towards enteropathogenic bacteria and are able to relieve from diarrhoea without causing adverse systemic immune or inflammatory outcomes. These probiotics are known to enhance immune function, reduces cholesterol levels, reduces the risk of diarrhoea, reduces the risk of eczema, relieves lactose intolerance symptoms and exhibits antitumorigenic activity (Marteau *et al.*,2001, Calder & Kew, 2002, Wright *et al.*, 2002, El-Shenawy *et al.*,2012,Mishra *et al.*, 2008). Because of these health benefits, efforts have been devoted to incorporate bifidobactria and Lactobacillus into dairy products and yoghurt containing probiotic cultures can prove a successful vehicle. Now a days, claims about the ability of foods, including herbs and spices, to lower disease risk or to enhance the quality of life continue to held interest on our lives (Kaefer C.M. and Milner J.A., Gupta P.,2015). However, properties of yoghurt such as pH and presence of oxygen and antimicrobial activity of herbal extracts can create a suboptimal environment for some probiotic species, reducing viability to below 10^6-10^8 colony forming units (CFU)/mL, which is the minimum accepted amount required to produce health benefits on the host (Reid *et al.*, 2001, Hekmat S *et al.*, 2009, Hemsworth J *et al.*, 2011, Behestipour H *et al.*, 2012 and Cimo A *et al.*, 2013). Therefore, it is important for the probiotic product fortified with herbal extract to serve the effective dose of probiotics required for health benefits, and for viable counts to be maintained throughout a standard product shelf-life of 28 days.

In the present study *Andrographis paniculata* nees (Acanthaceae) is used for fortification of milk which is a medicinal plant traditionally used for the treatment of anti-inflammatory, antibacterial, antioxidant, antiparasitic, antispasmodic, antidiabatic, anti carcinogenic, antipyretic, antidiarrhoeal, hepatoprotective, nematocidal, anti- HIV and several infectious diseases ranging from malaria to dysentery. The plant is widely used in ayurvedic and homeopathic systems of medicines. The medicinal value of this plant is due to the presence of active ingredients viz., andrographolide and neoandrographolide which are derivatives of diterpenoids. It prevents oxidative damage and inhibits binding to toxic metabolites to DNA. (Umadevi *et al.*,2013).

*Ocimum sanctum,*which is widely used in herbal medicines and as home remedy for treatment of many ailments. Polysaccharides isolated from *Ocimum sanctum* have antioxidant and radioprotective properties. *Ocimum sanctum* protects against various cancers particularly the breast cancer and reduces side effects of chemotherapy & radiotherapy. *Ocimum sanctum* inhibits growth & spread of various cancers such as breast cancer, liver cancer and sarcomas particularly fibrosarcoma by blocking supply of oxygen and nutrients to the cancer cells and killing them by starving. Ursolic acid isolated from *Ocimum sanctum* has immunoenhancing and tissue-protective properties (Umadevi *et al*.,2013). The present study investigated the effects of the herb on yoghurt fermentation, antioxidant activity and viability of probiotic bacteria during refrigerated storage.

MATERIALS AND METHODS

Procurement of cultures

the freeze dried culture of *Bifidobacterium bifidum* was obtained from NCBI, India and the locally isolated culture of *Lactobacillus acidophilus* from UGC project.

Preparation of extracts Powder of dry leaves of *kalmegh* and *Tulsi* was obtained from local resources. The powdered herbs (10g) were soaked in 100ml of distilled water and left overnight at 70˚C. The suspension was then centrifuged (2000 rpm; 15 min), and the supernatant was filtered.

Herbal yoghurt preparation

Homogenized and pasteurized buffalo milk was purchased from the local supermarket. Herb extract (6%v/v) was added in 100ml of milk. Inoculation was done using *Bifidobacterium bifidum* and *Lactobacillus acidophilus* culture (3% v/v). Incubation was carried out at room temperature for 18 hours then the samples were stored in refrigerator at 4˚C.

Determination of pH and TTA

The pH and TTA of yoghurts were determined during storage at 4˚C. Yoghurt sample (1g) was mixed with distilled water (1:1), and the pH was measured using a pH meter. TTA was determined by titrating yoghurt sample and distilled water (1:9) mixture with 0.1N NaoH using a 0.1% Phenolphthalein as colour indicator. The amount of acid produced during fermentation was calculated as follows:

TTA% = Dilution factor (10) x V NaoH x 0.1N x 0.009 x 100% where V is volume of NaoH required to neutralize the acid. (Behrad *et al.*, 2009)

Enumeration of probiotic bacteria

Enumeration of *Lactobacillus* spp and *Bifidobacterium* spp. was carried out by aseptically mixing yoghurt sample (1ml) with 9ml of distilled water and subsequently serial dilution technique was followed. Microbial count was carried out in agar plates of *Lactobacillus* spp. and *Bifidobacterium* spp. incubated anaerobically (CO_2 incubator) at 37˚C for 24-48 hours. Lactobacillus MRS agar (HiMedia) plates were used. For enumeration of *Lactobacillus* spp where as for enumeration of *Bifidobacterium* spp, BSC Propionate Agar (HiMedia) was used.

Viable microbial count was calculated as follows:

cfu/ml = cfu/plate x dilution factor (cfu :colony forming units)

Sensory properties evaluation

Yoghurt samples were evaluated for their sensory properties (appearence, aroma, consistency and flavour) on a 7-point hedonic scale (7-excellent, 6-liked a lot, 5-liked, 4-liked and did not liked, 3-disliked, 2-much disliked and 1-unacceptable) performed by expert judges selected and local resources. Yoghurt samples were presented in two-digit coded white plastic containers and tasted 20 minutes after leaving refrigerator.

Determination of syneresis

An amount of 10 g of the yoghurt sample was placed into centrifuge tube and centrifuged at 500rpm for 5 min and weighing the supernatant (guzman-gonzalez *et al.*, 2000). The weight fraction of the supernatant liquid was used as index of whey syneresis. Then measuring the amount of supernatant recovered (% v/w)

% synersis = (volume of supernatant x100)/ weight of sample

Antioxidant activity by 1,1-Diphenyl-2-Picrylhydrazyl (DPPH)

To 3ml of 60µM DPPH in ethanol, 250µl of each herba lyogurt water extracts was added and the decrease in absorbance was measured at 517 nm. The readings were compared with the controls, which contained 250µl of dH2O instead of the water extracts. The % of antioxidant activity inhibition was calculated as given in .Behrad *et al* (2009) :

$$\%\text{inhibition} = \frac{(A_{517}\,\text{control} - A_{517}\,\text{extract})}{A_{517}\,\text{control}} \times 100$$

RESULTS AND DISCUSSION

Effect of storage on pH

During the storage, in the *kalmegh* fortified yogurt the decrease in pH from 5.8 to 5.3 was observed constantly throughout the storage period (fig.1). This decrease might be attributed to the utilization of residual carbohydrates and production of lactic acid, small amounts of CO_2 and formic acid from lactose by viable microorganisms. (Panesar and Shinde,2011). On the other hand, the *Tulsi* fortified yogurt showed slight increase in pH from 3.8 to 4.2 during storage period of 21 days.

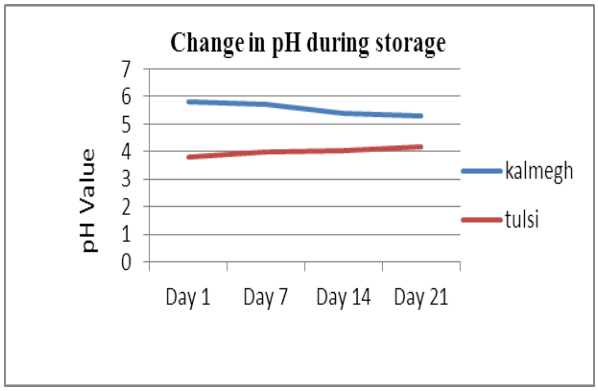

Figure 1: Effect of storage on Ph

Effect of storage on syneresis

Synersis was found to increase, in case of kalmegh yogurt from 38.5 to 74.0% during 28 days of storage as shown in fig.2. The increase in synersis may be due to increase in acidity i.e. decrease in pH throughout the storage period. Whereas,due to increase in pH during storage of *tulsi* yogurt, little decrease in synersis is obtained. These observations proves the theory of Fox *et al.*, (2000) which states that synersis is inversely related to pH.

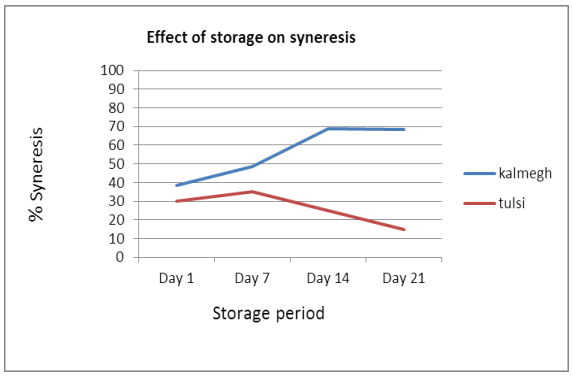

Figure 2.Effect of storage on synersis

Effect in Total Titrable Acid (TTA) of yoghurts during storage

The TTA of kalmegh yoghurt increased from the initial values of 0.45% to 0.72% and that of tulsi yogurt increased from 0.54% to 0.81% by day 21 of storage (fig.3). The increase in acids can be attributed to continued production of organic acids by LAB during refrigerated storage.

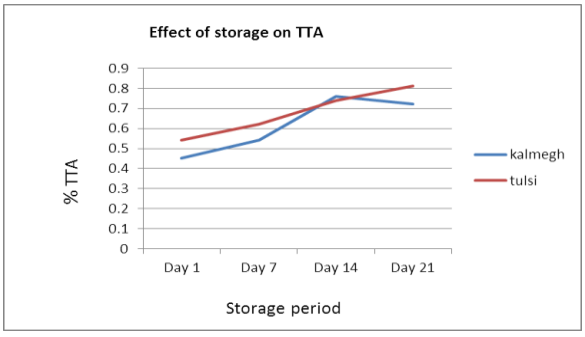

Figure 3.Effect of storage on TTA

Sensory properties.

The score of sensory properties viz. appearence, aroma, consistency and flavour on 7point hedonic scale are depicted in fig.4. Mean value of sensory properties scores are within the range of acceptance which depict it to be consumable.

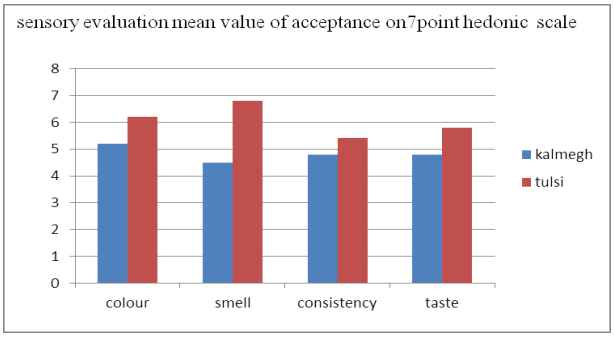

Figure 4.mean value score of sensory properties

Effect of storage on *Lactobacillus acidophilus* count

The changes in viable count of probiotics i.e., *Lactobacillus acidophilus* and *Bifidobacterium 3ebifidum* was monitored during manufacture and storage of *Kalmegh* and tulsi fortified probiotic yoghurt for 28 days in refrigerator at 4°C. During first week of storage an increase in count was observed from and then it decreased to the value of $14x10^8$cfu/ml in kalmegh yogurt and $55x10^8$cfu/ml in tulsi yogurt. It showed good viability till 21 days of storage (fig.5). This result are stastically at par with the findings of aloevera yogurt (Panesar and Shinde, 2011).

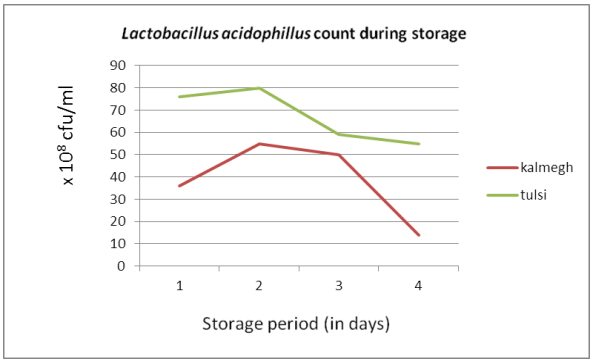

Figure 5.effect of storage on L.acidophilus count

Effect of storage on *Bifidobacterium bifidum* count

The changes in viable count of *Bifidobacterium bifidum* during manufacture and storage of *Kalmegh* fortified probiotic yoghurt for 28 days in refrigerator at 4°C are shown in fig.6. During the storage period the bacterial count decreases from $100x10^9$cfu/ml to $20x10^9$cfu/ml in kalmegh yogurt and $120x10^9$cfu/ml to $45x10^9$cfu/ml in tulsi yogurt. It showed good viability till 14 days of storage afterward a major decrease was noticed.

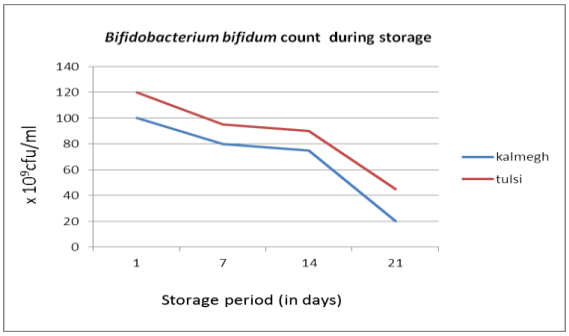

Figure 6.effect of storage on B.bifidum count

The viable counts of *Bifidobacterium bifidum* was found to be much more than *Lactobacillus acidophilus* throughout the storage period. The results indicate that *herbal* extracts may support the *Bifidobacterium* spp. more than *Lactobacillus* spp. The decrease in *Lactobacillus acidophilus* count may be due to antagonistic relationship between the probiotic strains and low dissolved oxygen content (Bari *et al.*,2009). However the counts of both the probiotics are more than the suggested value of 10^7 cfu/ml throughout the storage period. The results are in confirmation with Martin (1994) who found that probiotic can survive in sufficiently higher numbers to remain viable in cultured dairy products even during storage.

DPPH inhibition assay

The addition of *kalmegh* or *tulsi* increased the antioxidant activity of yogurts compared to plain-yogurt at all storage periods (Fig. 7). The highest antioxidant activity was recorded on day 7 for *tulsi*-yogurt (48.19 %) and on day 21 for *kalmegh*-yogurt (30.35%). These values are much more than that of plain-yogurt (21.04%). *Tulsi*-yogurt showed the highest antioxidant activity followed by kalmegh yogurt throughout the storage period.

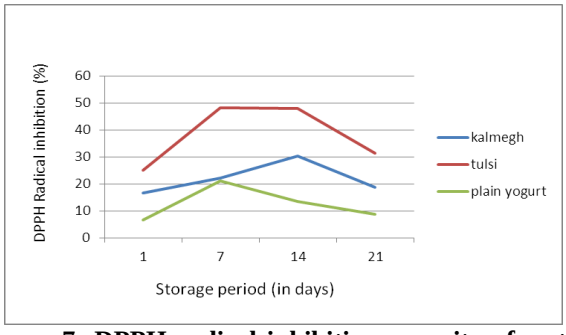

Figure 7: DPPH radical inhibition capacity of water extracts from plain and herbal-yogurts

CONCLUSION

Kalmegh and *Tulsi* fortified probiotic yoghurt has shown good viability of probiotic cultures i.e., *Bifidobacterium bifidum* and *Lactobacillus acidophilus* during storage period of four weeks. Viable counts of both the probiotics are more than the suggested value of 10^7cfu/ml throughout the storage period. Also, both the formulations are found to possess good antioxidant activity..The results suggest the potential of these herbal yoghurts for its commercialization as a functional food providing prevention from various infections and diseases.

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