Intl. J. Food. Ferment. 6(1): 1-10, June 2017

DOI: 10.5958/2321-712X.2017.00001.1

Supplementation of milk with honey, oat, *safed musli*, carrot juice and its effects on characteristics and shelf-stability of probiotic products

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Abstract

The current work is aimed to study the effect of supplementation of milk on culture viability, characteristics and shelf life of probiotic fermented milk products. The products were prepared using toned milk supplemented with honey, oat, *safed musli* (*Chlorophytum borivilianum*), carrot and fermenting with probiotic *Lactobacillus helveticus* MTCC 5463 and *Streptococcus thermophilus* MTCC 5460. Probiotic fermented milk without any supplementation served as control. Products were evaluated periodically for sensory properties, probiotic and starter viability, pH, free fatty acids and tyrosine content during storage at 5±2°C. Supplementation had a significant effect on sensory, culture count and chemical properties of products. Sensorily all products exhibited significant difference in flavor, colour and appearance, body and texture and overall acceptance till the end of storage period. Honey containing product was the most acceptable sensorily. All products exhibited an increasing trend in culture counts till 14 days of storage, after which the counts started declining. Probiotic viability was highest in carrot followed by honey, oat, *safed musli* compared to control. All products had >9log cfu/g probiotic count at the end of 21days of storage. In comparison with control the increase in free fatty acids and tyrosine were significant in all products and this increase was found to be highest in oat and musli containing products. Over all, a positive effect of supplementation was observed on the probiotic count and sensory aspects of products.

Keywords: *Lactobacillus helveticus* MTCC 5463, probiotic viability, supplementation, shelf-stability, sensory properties

Fermented milks are the widely accepted format for delivery of health benefitting microbes popularly called probiotics. The matrix of fermented milk affects probiotic survival, physiology, and its potential efficacy. The growth of probiotics in food matrices is found to be enhanced in the presence of bioactive ingredients such as prebiotics, vitamins, minerals, etc. (Sanders and Marco 2010). Honey contains 4 to 5% fructooligosaccharides which serve as prebiotic agents (Chow 2002). A number of studies have proved the prebiotic potential of honey in terms of increase in viable count of probiotic lactobacilli and bifidibacteria when used along with skim milk (Mei *et al.*, 2010; Hosny *et al.*, 2009; Riazi and Ziar 2008; Macedo *et al.*, 2008). Oat grains are good source of minerals, antioxidants, B-complex vitamins and soluble fiber β-glucan. The health benefits of oat are highlighted in a number of research works (Wood 2007; Butt *et al.*, 2008; Immerstrand *et al.*, 2010; EFSA 2009). Carrots posses many reported health benefits owing to the presence of beta carotene, fibres, B-group vitamins, minerals and bifidogenic factors (Christensen and Brandt 2006; Moorhead *et al.*, 2006). *Safed musli* (*Chlorophytum borivillianum*) is reported to be a rich source of alkaloids, vitamins, seponins, minerals, polysaccharides, etc and has found application in traditional ayurvedic medicine (Haque *et al*., 2011; Agrawal *et al*., 2013). Taking these points into consideration, in the current work, effects of supplementation of milk with honey, oat, *safed musli* and carrot on fermented product quality, probiotic viability and shelf life of probiotic *lassi* products were studied.

MATERIALS AND METHODS

Bacterial cultures

The cultures, *Streptococcus thermophilus* MTCC 5460 and probiotic strain *Lactobacillus helveticus* MTCC 5463 were obtained from the culture collection of Dairy Microbiology Department, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India. Both the cultures were maintained by propagating in sterilized reconstituted skim milk (10% Total Solids) and stored at 5±2ºC. *Lactobacillus helveticus* MTCC 5463 has been proved for its probiotic properties (Ashar and Prajapati 1998; Khedekar *et al*., 1990; Ashar and Prajapati 2000; Prajapati *et al*., 2011; Vishwanath *et al*., 2012). *Streptococcus thermophilus* MTCC 5460 is a *dahi* isolate, fully characterized and studied extensively for its application in fermented milks especially *dahi*, *lassi* and yoghurt either alone or in combination with lactobacilli.

Raw materials

Toned milk was purchased from a commercial dairy plant. High quality sugar, free from impurities was purchased from local market. Processed honey from a specific reputed brand was purchased from local market. Oat flour used was supplied complimentary by Ceba foods, AB, Lund, Sweden. Fresh carrots were procured from local market and carrot juice was prepared using electrical juicer (National Co. Pvt. Ltd., Mumbai, India). Complimentary extract powder of *safed musli* was supplied by M/S Green Chem Ltd. Bangalore, India.

Method for preparation of probiotic fermented milk

Standard procedure for probiotic fermented milk making with minor modification of the procedure adopted by Patidar and Prajapati (1998) was used. Fresh toned milk having minimum 3.0 % fat and 8.5 % solids not fat (SNF) was used for preparation of probiotic *lassi*. Pre-warmed (45ºC) toned milk was supplemented with (i) Honey @ 5% (ii) Oat @4% (iii) *Safedmusli*@1% and (iv) Carrot in the form of juice @15%(v/v) as per the type of the product. Sugar was added at the rate of 5% for product with honey while for carrot, oat and *safed musli* 10% was added. All the rate of additions were based on works carried out previously in the department. After proper mixing, a heat treatment of 90 ºC for 5 min. was given to the mixture followed by cooling to 40 ± 2 ºC. Active cultures of *S. thermophilus* MTCC 5460 and *Lb. helveticus* MTCC 5463 were inoculated @1% v/v each and stirred well. The inoculated milk was incubated at 37 ± 2ºC till the acidity reached to 0.65 % lactic acid. The set curd was immediately transferred to refrigerator to arrest further acid production. After allowing it to cool to 10ºC, the curd was broken by mechanical stirrer to get probiotic fermented milk. The product was then filled in polyethylene cups with lids and stored at refrigeration temperature (5±2ºC) for shelf-life study. The products were analyzed for sensory, microbiological, chemical and compositional parameters.

Storage studies of probiotic products

The products were stored at refrigeration temperature (5 ± 2°C) to decide shelf life and to study the storage related changes in the products. Products were analyzed for proximate composition at the day of preparation, while analysis of free fatty acids and tyrosine value were performed along with measurement of acidity and pH at 7, 14, 21, 28 days intervals. Samples were also evaluated for sensory and microbiological parameters at all intervals.

Analysis of products

Sensory evaluation

The products were subjected to sensory evaluation by an expert panel of nine judges for colour and appearance, flavour, body and texture and overall acceptability criteria. Fresh products and the stored products (7, 14, 21 and 28 days storage at 5 ± 2 °C) were brought to 10 °C before giving to judging. The score given by them on 9 point hedonic scale (9 liked extremely to 1 disliked extremely) were taken to determine the acceptability level of product.

Chemical analysis

The products were analyzed for chemical parameters such as titratable acidity, moisture, fat, ash, total carbohydrates and total protein (ISI Handbook of Food Analysis 1989), Free Fatty Acids (FFA) (Deeth and Fitz-Gerald 1976) and Tyrosine Value (Hull 1947). pH was determined by electronic pH meter (Model CYBERSCAN 2100, EUTECH Instruments, Singapore).

Microbiological analysis

Fermented milk samples for microbial analysis were prepared by aseptically transferring eleven grams of sample to 99ml sterile phosphate buffer to obtain 1:10 dilution. Subsequently, 1 ml of the said dilution was used for making further dilutions in 9 ml phosphate buffer. Suitable dilutions were prepared and poured in a set of sterile Petri dishes in duplicates. Microbial counts were determined as per standard procedures. Lactobacilli count on MRS agar (De Man *et al*. 1960), Streptococci count on M17 agar (IS: 1479, Part III 1962), Yeast and Mold count (IS: 5403 1969) and Coliform count (IS: 5401 1969) were estimated. The media and chemicals were purchased from Hi Media, Mumbai, India. All chemicals used in this study were of analytical grade.

Statistical analysis

Optimization study data were analyzed by Completely Randomized Design (CRD) as per the methods described by Steel and Torrie (1980). Storage study data were examined using Factorial CRD. The values for microbial counts were log transformed before analysis.

RESULTS AND DISCUSSION

Proximate composition of the probiotic products are shown in Table 1. Overall compositional analysis indicated that protein and total solids were higher in oat containing product as compared to other products.

Sensory evaluation of the products

All the products were subjected to sensory evaluation by a panel of expert judges using nine point hedonic scale. The scores obtained are shown in Table 2. Significant changes were seen in the color and appearance, flavor, body and texture and overall acceptability scores of all products. The scores for sensory parameters showed a declining trend with increase in storage period. This decrease was more in case of oat and *musli* containing products. Patidar and Prajapati (1998) reported a similar decrease in the sensory scores of probiotic *lassi* samples during refrigerated storage. Gawai and Prajapati (2012) reported that synbiotic products such as synbiotic *raita*, synbiotic *lassi* remained organoleptically acceptable even after a storage period of 3 to 4 weeks at refrigeration temperature. At the end of storage period of 28 days, the overall acceptability scores for all products except product with honey, were found to be <5.0 on nine point hedonic scale. But the scores for overall acceptability of all products were >5.5 after 21 days of storage. Hence, the shelf life of products were concluded as 21 days at 5 ± 2 °C. Honey containing product was rated the best for sensory parameters followed by carrot, control, oat and *safed musli*. Product with honey scored more than 6.5 on hedonic scale rating even after 28 days of storage at refrigerated temperature. Honey is said to have a flavor enhancing effect in fermented milk in addition to its proposed prebiotic effect. Improved sensory parameters of yoghurt fortified with honey were reported by Ghadge *et al*. (2008) and Varga (2006) in their studies.

Microbial profile of products during storage

A high level of probiotic cell count at the end of product shelf-life is a must for ensuring functionality of the product and hence maintenance of viability of probiotics during storage becomes one of the main requirements for a probiotic product. At the same

Table 1: Proximate composition of probiotic *lassi* products

Probiotic *lassi*	Constituents (%)					
	Moisture	Total solids	Fat	Protein	Carbohydrate	Ash
Control	78.88±0.85	20.82±0.75	3.00±0.02	3.54±0.15	12.81±0.55	0.67±0.05
Honey	77.54± 0.91	22.67±0.39	3.00±0.0	3.56±0.12	15.04±0.93	0.84±0.06
Carrot	76.79±0.91	23.39±0.94	3.00±0.0	3.43±0.08	16.11±0.90	0.69±0.02
Oat	75.3±2.10	25.68±2.48	3.00±0.0	4.26±0.18	16.69±1.92	0.79±0.08
Safed musli	78.18±0.75	21.48±0.38	3.03±0.05	3.50±0.04	14.80±0.60	0.72±0.04

Each observation is mean±SD of four replications

Table 2: Effect of storage on the sensory aspects of probiotic *lassi* products based on 9-point hedonic scale

Sensory parameter	Storage period (days)				
	0	7	14	21	28
Colour and appearance scores out of 9.0					
Control	8.08±0.14	7.49±0.02	7.24±0.12	6.82±0.04	5.09±0.09
Honey	8.62±0.10	8.20±0.08	8.03±0.17	7.83±0.15	6.00±0.0
Carrot	8.25±0.17	7.93±0.19	7.70±0.20	7.25±0.19	5.90±0.08
Oat	8.00±0.11	7.40±0.23	7.15±0.10	6.70±0.26	4.88±0.32
Safed musli	8.25±0.10	7.83±0.05	7.28±0.30	6.55±0.13	5.05±0.06
CD (0.05) S=0.141, P=0.126; SxP=0.281					
Flavour scores out of 9.0					
Control	8.28±0.06	7.69±0.06	7.34±0.10	6.42±0.14	5.59±0.11
Honey	8.65±0.06	8.13±0.10	8.08±0.05	7.48±0.19	5.93±0.10
Carrot	8.40±0.14	7.83±0.13	7.65±0.10	7.05±0.06	5.70±0.14
Oat	7.78±0.39	7.15±0.30	6.95±0.17	5.95±0.19	3.05±0.10
Safed musli	8.15±0.13	7.55±0.19	7.25±0.17	6.15±0.19	3.53±0.47
CD (0.05) S=0.121, P=0.108; SxP=0.242					
Body and texture scores out of 9.0					
Control	8.01±0.14	7.40±0.10	7.06±0.07	6.59±0.15	5.68±0.06
Honey	8.43±0.17	8.10±0.14	7.98±0.05	7.53±0.10	5.98±0.13
Carrot	8.13±0.13	7.85±0.10	7.58±0.05	7.05±0.25	5.60±0.18
Oat	7.73±0.22	7.33±0.17	7.10±0.11	5.55±0.30	3.00±0.0
Safed musli	8.00±0.0	7.73±0.15	7.33±0.10	6.20±0.29	3.68±0.54
CD (0.05) S=0.140, P=0.125; SxP=0.281					
Overall acceptability scores out of 9.0					
Control	7.85±0.20	7.53±0.16	7.06±0.10	6.25±0.22	4.62±0.20
Honey	8.58±0.10	8.28±0.31	8.00±0.08	7.35±0.13	6.50±0.38
Carrot	8.08±0.22	7.83±0.05	7.28±0.24	6.70±0.26	4.75±1.50
Oat	7.80±0.0	7.25±0.34	6.55±0.06	5.95±0.71	3.88±1.11
Safed musli	7.75±0.06	7.43±0.53	6.88±0.33	5.61±0.31	3.25±0.29
CD (0.05) S=0.358, P=0.320; SxP=0.716					

Each observation is mean±SD of four replications, S= Storage period, P= Sensory Parameter.

time the viable count of starter culture and their activity during storage, interaction of the probiotic and the starter cultures are important determinants of probiotic survival and product quality during storage. Additionally, the presence of contaminants, mainly coliforms, yeast and molds can bring spoilage of the product. Coliforms are opportunistic pathogens, which generally indicate hygiene and post pasteurization contamination. Dave (1991) reported that coliforms are not able to thrive in acidic products; moreover the antagonistic effects of starter cultures inhibit their growth. Yeasts and molds are indicative of aerial contamination and also could grow in presence of acidic environment in fermented milk. Their growth and subsequent development of off-flavors can be a major cause of spoilage of fermented products and thereby influencing the shelf-life of the product.

The changes in probiotic count of fermented products stored at refrigerated temperature are shown in Fig. 1. The average probiotic count differed significantly ($P<0.05$) among the probiotic products. This could be because some food matrices might be better than others for ensuring high amounts of viable probiotic cells (Sanders and Marco 2010). A gradual increase in probiotic count upto 14 days of storage was observed in case of all products. This increase was highest in case of product containing carrot (10.42 log cfu/g) followed by honey (10.18 log cfu/g), *musli* (10.06 log cfu/g), oat (9.99 log cfu/g) and control (9.98 log cfu/g).

Afterwards the counts decreased in all the products during storage up to 28 days. This could be due to the fact that during the manufacturing process the culture count increase in number and continue to multiply for about few more days. Additionally, probiotics were found to survive better in food products added with prebiotic ingredients. Additions of inulin, high amylase corn starch powder, or fructooligosaccharides improved the survival of probiotic cells in dairy products (Desai *et al*. 2004; Capela *et al*. 2006; Varga *et al*. 2006; Donkor *et al*. 2007). Boeni and Pourahmad (2012) reported an increase in counts of probiotic bacteria in synbiotic yoghurt until 7th day of storage at 4^{0}C followed by a decrease in count which was not significant. Rayes (2012) reported a similar trend in their study, where the numbers of *B. bifidum* were increased in all treatments and reached maximum in fermented milk made with honey after 5 days of storage. A decrease in viable count of probiotic lactobacilli was reported by Patidar and Prajapati (1998) for the *lassi* stored in polyethylene pouches and glass bottles until storage period of 6 days, but later on it increased to some extent. In the current study, probiotic viability of >9log cfu/g was observed in all products till the end of 28 days of storage (Fig. 1). Gawai and Prajapati (2012) reported that synbiotic products such as synbiotic *raita*, synbiotic *lassi* contained optimum dose of probiotic lactobacilli even after storage period of 3 to 4 weeks at refrigeration temperature. Shaghaghi et al (2013) also reported good viability of probiotic strains in synbiotic yoghurts even after storage of 28 days at 4°C. The current study results showed that the added supplements has a significant effect on the probiotic count in the products and can serve as suitable delivery vehicle for probiotic lactobacilli.

Significant ($P<0.05$) changes in the streptococcal count of probiotic products were observed during storage period (Fig. 2). High streptococci counts were found in carrot as well as honey products compared to oat, *musli* and control. The streptococcal counts increased till 14 days of storage for all the products (mean count ranged from 9.59 to 10.13 cfu/ g of product) and subsequently a decrease (mean count 9.66 cfu/ g of product) was observed. The interaction effect of the treatments with the periods also showed significant changes.

Similar trend in the counts of lactobacilli and streptococci strains was reported by Patidar and Prajapati (1998) in *lassi* and by Hosny *et al*. (2009) in yoghurt fortified with honey during storage study. Coliforms and yeast and mold were absent during storage period in all the products. The absence of coliforms and yeasts and molds in fermented milk products stored at refrigerated conditions were also reported by other workers (Patidar and Prajapati 1998; Al-Otaibi 2009; Rayes 2012).

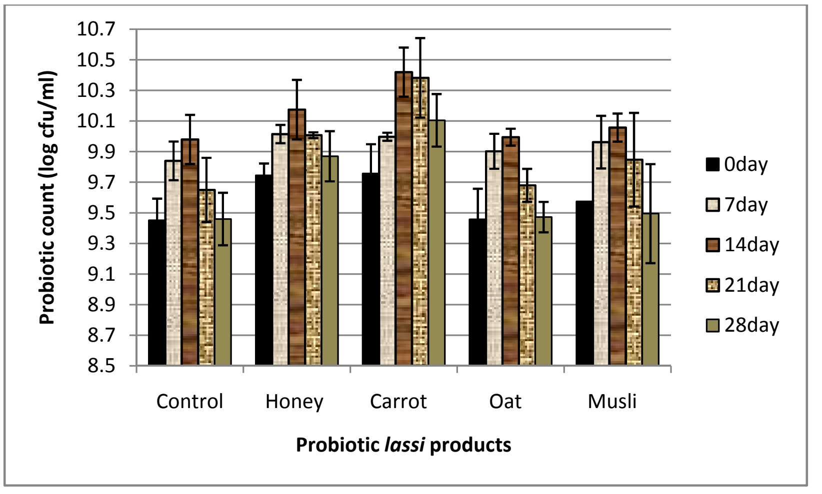

Fig. 1: Changes in the probiotic count of *lassi* products during storage at 5±2°C

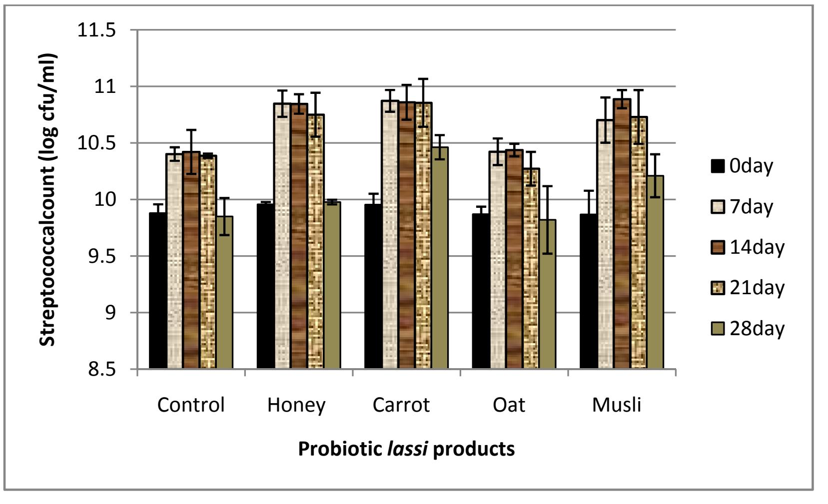

Fig. 2: Changes in the streptococcal count of probiotic *lassi* products during storage at 5±2°C

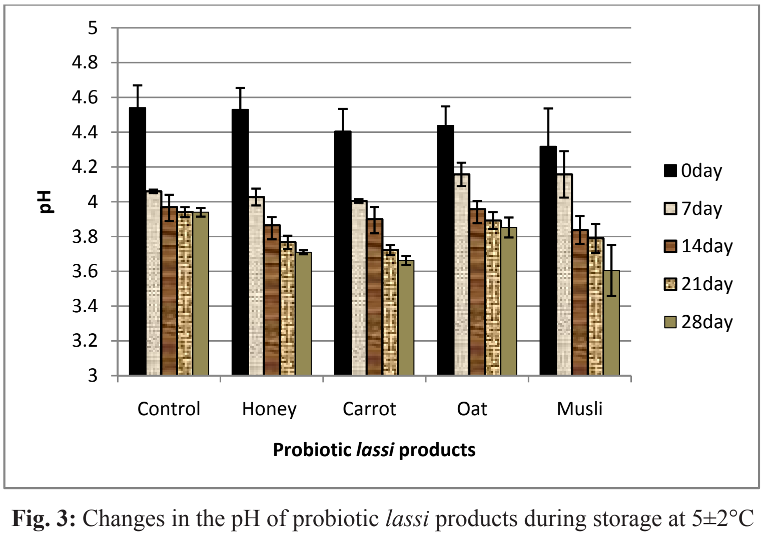

Fig. 3: Changes in the pH of probiotic *lassi* products during storage at 5±2°C

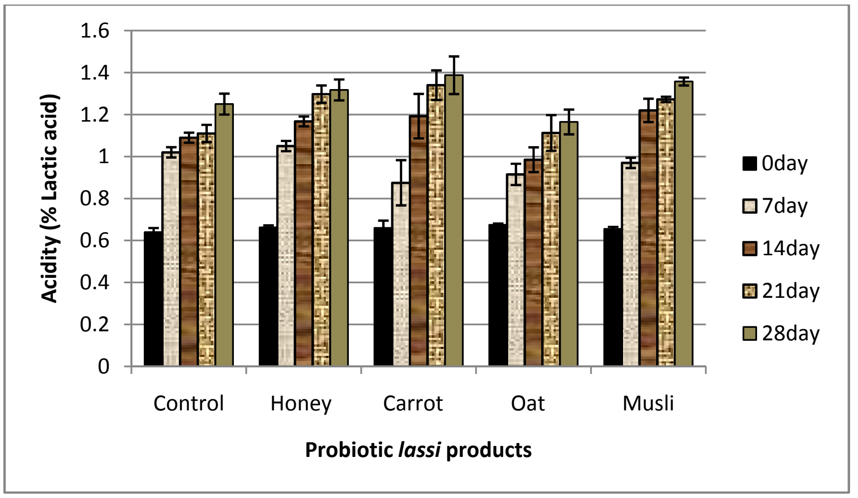

Fig. 4: Changes in the acidity of probiotic *lassi* products during storage at 5±2°C

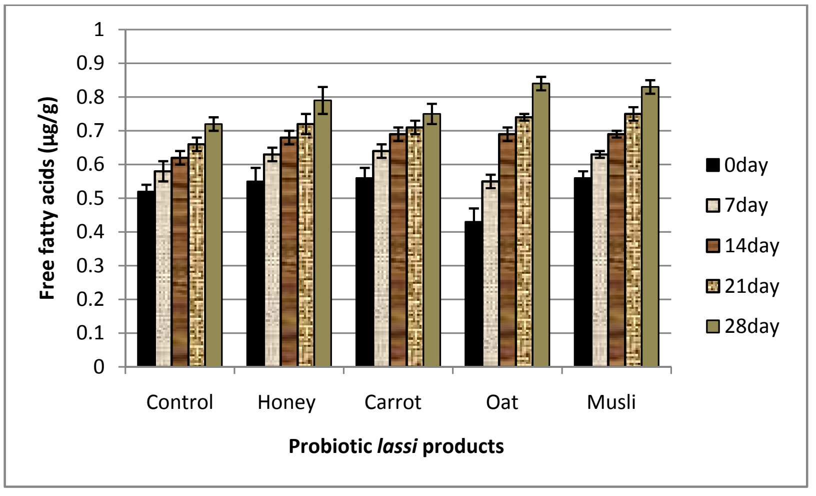

Fig. 5: Changes in the FFA content of probiotic *lassi* products during storage at 5±2°C

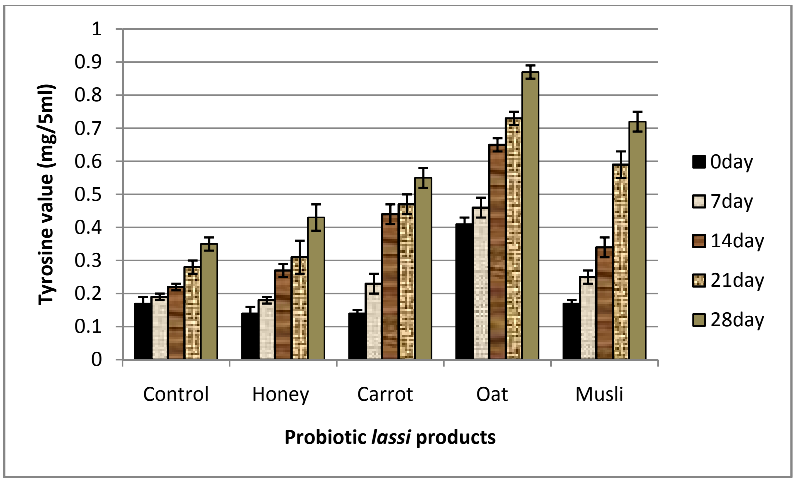

Fig. 6: Changes in the tyrosine content of probiotic *lassi* products during storage at 5±2°C

Chemical changes in the products during storage

Changes in pH and acidity

The chemical parameters of the products such as pH, acidity, FFA, tyrosine content are all indicators of the chemical changes happening in the products during storage. These changes determine the sensory, physical and chemical qualities of the products during storage. Hence, understanding these changes is important for determining the shelf life of the products. The changes in pH and titratable acidity (% Lactic acid) of the products during storage are shown in Figures 3 and 4 respectively. The changes were significant between the products and for different storage periods. A gradual decrease in pH and a corresponding increase in acidity was seen in all products. This increase was less in case of oat containing product compared to others. Al-Otaibi (2009) reported a significant decrease in the pH values of commercial probiotic fermented milk products from the production day to the end of storage period. Boeni and Pourahmad (2012) reported a similar decrease in pH value of synbiotic yoghurt when stored at 4°C for three weeks. Such declining trend in pH values of synbiotic yoghurts during storage was reported by Hekmat *et al.* (2009), Ramchandran and Shah (2010), Mazloomi *et al.* (2011) and Shaghaghi *et al*. (2013). Rayes (2012), reported a gradual increase in the acidity values along the storage period in fermented milk made without or with prebiotic substances honey or inulin and different types of probiotic strains.

Changes in the FFA content

A gradual and significant (P<0.05) increase in the FFA content of all products were seen during the storage period (Fig. 5), which is indicative of the lipolysis happening in the products due to the lipolytic activity of the cultures used in the study. The lipolytic enzymes, lipases and esterases may have acted upon the fat and released the fatty acids. Mean FFA value ranged from 0.52 to 0.81 µg/g during storage period. The changes were more pronounced in case of oat and *musli* containing products. Yadav *et al.* (2007) studied the production of fatty acids and conjugated linoleic acid (CLA) in the ordinary and probiotic yogurt containing *L. acidophilus* and *L. casei*, during fermentation and after 10 days storage at 4˚C. They reported an increased level of fatty acids during fermentation and storage in the probiotic yogurt samples mainly due to lipolysis of milk fat. Vaseji *et al.* (2012) also reported an increase in levels of butyric acid in probiotic yogurt samples with increased shelf life.

Changes in the Tyrosine content

Tyrosine value of all products increased significantly (P<0.05) during storage (Figure 6). The mean tyrosine value ranged from 0.23 to 0.67 mg/5ml during storage period. Tyrosine value is a measurement of the proteolytic action going on in the products due to action of cultures and serve as an indicator of chemical deterioration of the products. The proteolytic change was more pronounced in case of oat (mean value 0.64 mg/5ml) and *musli* (mean value 0.43 mg/5ml) containing products compared to honey (mean value 0.29 mg/5ml) and carrot (mean value 0.39 mg/5ml). The increase in tyrosine content of fermented milk products dependent on storage time, culture type and protein structure was reported by Kesenkaş *et al.* (2011) and Costa *et al.* (2015).

CONCLUSION

The study results suggest that supplementation of milk with honey, carrot, oat and *safed musli* had a positive effect on the probiotic count as well as the sensory aspects of products. All the probiotic fermented products were comparative in terms of the probiotic count (>9 log cfu/g) even at the end of storage period of 28 days and thus fulfilling the requirement of delivering a large number of viable probiotic strain through these products. Honey and carrot containing products were superior in terms of sensory and chemical aspects as compared to oat and *musli* containing products. Fermented product with honey scored maximum for sensory parameters may be due to the flavor enhancing effect of honey. All the products were liked by judges till the end of shelf life of 21 days when stored at 5 ± 2 °C. These products can serve as a good carrier as well as delivery vehicle for probiotics. These probiotic products should be further researched for their functional properties.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge the financial support provided by Department of Biotechnology (DBT), New Delhi, India.

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