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DEVELOPMENT OF ANTIOXIDANT RICH FRUIT FORTIFIED PROBIOTIC BUTTERMILK (LASSI) USING *LACTOBACILLUS RHAMNOSUS* CULTURE

KUMAR A AND KUMAR D*

School of Bioengineering and Food Technology, Shoolini University, Solan, India

***Corresponding author: Dr. Dinesh Kumar: Professor & Head, School of Bioengineering and Food Technology, Shoolini University, Solan-173212, H.P., India; Mobile No.: +91 9816321855; Ph. Office. 01792 308000; Fax: 01792 308000: E-mail: dineshkumar@shooliniuniversity.com**

ABSTRACT

The present study reports the preparation of fruit fortified probiotic buttermilk (lassi) using *Lactobacillus rhamnosus*. Probiotic lassi was prepared by supplementing commercial lassi (Verka, India) with probiotic *Lactobacillus rhamnosus* (5%). Probiotic lassi samples were fortified with antioxidant rich fruit juice (10% v/v) of *Rubus ellipticus*, *Prunus domestica*, *Prunus armeniaca* and *Syzygium cumini*. The probiotic lassi samples were prepared using free, alginate (2%) and carrageenan (2%) encapsulated probiotic culture. The microencapsulated beads were characterized by FTIR technique wherein the intensity of peak increased when bacterial cells were entrapped within the matrix. The acidity of lassi samples increased (0.56 ± 0.02 - 0.84 ± 0.02%) and pH decreased (3.92 ± 0.05 - 2.40 ± 0.05) continuously during 15 days of storage. Probiotic *Lactobacillus rhamnosus* count decreased during storage, however, alginate microencapsulated culture was more stable as compared with carrageenan encapsulated and free culture. Apricot and raspberries fortified lassi prepared using alginate encapsulated probiotic culture retained recommended probiotic values of 6.42±0.03 and 6.55±0.03 log CFU/ml respectively up to 5 days of storage. The antioxidant power of fruits fortified probiotic lassi samples decreased successively during storage. In DPPH radical scavenging and NORS assay the percentage scavenging decreased from 64.22±0.86 to 38.93±1.59 and from 65.60±1.62 to 39.29±3.29 respectively. In FRAP assay the optical density decreased from 0.657±0.02 to 0.423±0.01 during storage up to 15 days.

Keywords: Probiotic *Lactobacillus rhamnosus*, microencapsulation, fruit fortified probiotic buttermilk, antioxidant analysis

INTRODUCTION

The relationship between certain food and its health benefits has been investigated from many years. The development of foods that confer health benefits is the key research priorities of food industry [1]. Metchnikoff [2] pointed out the use of fermented milks for prevention of certain diseases of gastrointestinal tract. A number of studies have shown that the fermented food products do have a positive effect on health status [3]. Fermented foods are more nutritious than unfermented ones and foods prepared by using lactic acid bacteria (LAB) have better acceptability [4]. Buttermilk (lassi) is a fermented drink prepared by churning of the curd. The functionality of lassi increases with the addition of probiotic microorganisms and incorporation of antioxidant rich fruit juice provides strong antioxidant power endow with value addition to the finished product.

Probiotics are live microorganisms that when administered in adequate amount confer a health benefit on the host [5]. Several health benefits associated with the consumption of live probiotic bacteria are in controlling intestinal infections, improved digestion, improved lactose utilization, prevention of colon cancer, lowering of blood pressure, cholesterol and reduced inflammation etc. [6-9]. Consumption of fruits has significant health promoting effects and reduces the incidence of cardiovascular diseases, cancer and various degenerative diseases [10-12]. Himachal Pradesh is known as the fruit bowl of India and most of the wild fruits are underutilized in the state and can be used for the development of fruit fortified probiotic lassi. Wild fruits viz. raspberries (*Rubus ellipticus*), plum (*Prunus domestica*), apricot (*Prunus armeniaca*) and jamun (*Syzygium cumini*) were found rich in antioxidants as reported in our previous publication [13]. The free radicals are considered to play a causative role in aging and several degenerative diseases e.g. heart disease, cataracts, cognitive dysfunction and cancer [14-15]. The antioxidants are the major defense system that prevents the body from damage by neutralizing the free radicals [16].

Probiotics bacteria must arrive in intestines alive in sufficient numbers i.e. 6-7 log CFU/g of products to confer health benefits. The probiotic products face the problem of variation in viability of cultures in various developed products. The viability of microbial cells can be improved by using microencapsulated probiotic culture. Microencapsulation of probiotic microorganisms with alginate or other gels generally improves survival of probiotics in food products [13, 17-19]. Keeping in view

the potential of probiotics and fruit antioxidants, the present study is endeavoured to develop antioxidant rich fruit fortified probiotic buttermilk using free and microencapsulated probiotic *Lactobacillus rhamnosus* culture.

MATERIALS AND METHODS

1. Bacterial Strain

The probiotic *Lactobacillus rhamnosus* LBS 2 culture was isolated and characterized as reported in our earlier publications [20-21].

2. Microencapsulation, FTIR spectroscopy analysis and viability count of *L. rhamnosus*

Microencapsulation of the *Lactobacillus rhamnosus* was performed according to the method described by Vodnar *et al.* [22] using alginate (2% w/v) and carrageenan (2% w/v) as described earlier in our publication (Kumar and Kumar, 2016). Briefly, the alginate emulsion containing bacterial suspension was passed through the bioencapsulator (Buchi-390, India) nozzle (0.3mm) into sterile solution of $CaCl_2$ (2% w/v) and the carrageenan emulsion containing bacterial suspension was passed through syringe needle (0.5mm) in to KCl (2% w/v) for stabilization of microcapsules. The microencapsulated beads were characterized using FTIR spectroscopy. For viability count briefly, 1 g of each bead type was dipped in 99 ml sodium citrate 1% (w/v) at pH 6.0 and stirred continuously for 20 min at 150 rpm. The bacteria released from beads were counted on MRS (de Man, Rogosa, and Sharpe) agar [22].

3. Antioxidant analysis of fruits selected for fortification of buttermilk (lassi)

Fruits viz. wild apricot (*Prunus armeniaca*), wild raspberries (*Rubus ellipticus*) and damson plum (*Prunus domestica*) were collected during May-September from Solan in Himachal Pradesh, India. Jamun (*Syzygium cumini*) fruits were collected from Hamirpur in Himachal Pradesh, India. Different antioxidant analysis viz. 2, 2-diphenyl-l-picryl hydrazyl (DPPH) radical scavenging assay [23], ferric reducing antioxidant power (FRAP) assay [24] and nitric oxide radical scavenging (NORS) assay [25] were performed as described earlier by Kumar and Kumar [13]. Ascorbic acid (20–100 µg/ml) was used as a standard.

4. Preparation of antioxidant rich fruit fortified probiotic buttermilk (lassi)

Probiotic buttermilk was prepared using commercial lassi (Verka, India). Three sets of buttermilk viz. probiotic buttermilk without fruits and jamun, apricot, raspberries and plum juice fortified buttermilk were prepared using free, alginate and carrageenan encapsulated *L. rhamnosus* culture. In first set, buttermilk

was divided in to five parts (First part 95 ml and 2-5 parts were 85 ml each). Probiotic *Lactobacillus rhamnosus* LBS 2 culture (5% v/v containing 10^7-10^8 CFU/ml) was added to all parts. First part was left unaltered and pasteurized juice of selected fruits viz. jamun, apricot, plum and raspberries (10% v/v) was added to 2-5 parts followed by homogenization and stored at 4°C for further investigation. In second set, probiotic buttermilk with and without fruit supplements were prepared in similar way as described above by replacing the free culture with alginate encapsulated probiotic culture (5% w/v containing 10^7-10^8 CFU/g). In third set, probiotic buttermilk samples was prepared using carrageenan encapsulated probiotic culture (5% w/v containing 10^7-10^8 CFU/g) and investigated further.

5. Storage stability study of the buttermilk (lassi)

All probiotic buttermilk samples were analyzed for pH, acidity and selective enumeration of probiotic microorganisms on 1, 5, 10 and 15 days of storage at 4ºC.

pH and Acidity

The pH value of the lassi samples during storage was recorded with digital pH meter (Deluxe pH meter, India). The titratable acidity (% lactic acid) was determined after mixing lassi samples with 10 ml of distilled water and titrating with 0.1 N NaOH using 0.5% phenolphthalein as indicator [26].

Selective enumeration of probiotic *Lactobacillus rhamnosus* in lassi

Selective enumeration of *L. rhamnosus* was performed according to the method described by Saccaro *et al.* [27]. MRS - vancomycin hydrochloride (MRS-V) agar media was used for selective enumeration. Briefly, 50 µl of vancomycin solution (100 mg in 5 ml w/v) was added to 100 ml of sterile MRS agar just before pouring the plates at 40°C. CFU/ml was calculated using standard serial dilution method, serial dilutions of 1 ml of lassi samples were prepared in peptone water in case of free probiotic culture and for microencapsulated culture the serial dilutions were prepared in sodium citrate (1% w/v). After this, 100 µl samples from different dilutions were spread over the solidified MRS-V medium and incubated at 37□C for 48 h [28].

6. Antioxidant analysis of finished products during storage at 4ºC

Antioxidant analysis of the fruit fortified buttermilk was performed during storage period (1-15 days) at an interval of 5 days. Briefly, 4% (v/v) of antioxidant rich fruit supplemented lassi samples were used for the antioxidant analysis. Different antioxidant tests viz. DPPH radical scavenging assay, FRAP assay and NORS assay were performed as per the protocols

described in detail in our previous publication [13]. Probiotic lassi without fruit supplements was used as a negative control.

7. Statistical analysis

All data were expressed as mean ± S.D. Data were analyzed by means of analysis of variance, average and standard error using GraphPad Prism 5.0. A p value of < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

1. Microencapsulation, FTIR spectroscopy and viability count of *L. rhamnosus*

Probiotic *L. rhamnosus* culture was microencapsulated in alginate and carrageenan matrix to evaluate its survival in buttermilk (lassi). Alginate and carrageenan beads were round in shape and the alginate beads prepared using Bioencapsulator were 0.3 mm in size and carrageenan beads size ranged from 0.5-1 mm. The comparative FTIR spectrum of encapsulated bacteria in alginate and carrageenan beads, free bacteria and free beads was obtained. The intensity of the peak increased when bacteria were encapsulated in the alginate or carrageenan [13]. The entrapment of bacteria in beads containing 7-8 log CFU/g was also confirmed by colony count method. Ayama *et al.* [29] encapsulated *Lactobacillus plantarum* CM53 in 1-3% alginate and 2% Hi-maize resistant starch. The encapsulation of probiotic cells in k-carrageenan beads was done by Dinakar and Mistry [30]. Kebary *et al.* [31] encapsulated *Bifidobacterium bifidum* and *B. infantis* in alginate and k-carrageenan (3%) using syringe needle. Similar study was also done by Buyukgungor [32]. Vodnar *et al.* [22] reported FTIR study of the free bacterial suspension, free beads and beads encapsulated bacteria and results were comparable to the present findings.

2. Antioxidant analysis of selected fruits

All selected fruits were analyzed for antioxidant potential before fortification in products. Detailed description of antioxidant analysis of selected fruits before their supplementation is reported earlier [13]. Briefly, in DPPH radical scavenging assay, IC_{50} value of apricot, raspberries and plum fruits was observed in the concentration range of 200-400µg/ml. However, in jamun fruit IC_{50} value was <200 µg/ml. In FRAP assay a linear increase (0.391±0.01-0.698±0.02%) in reducing power of all fruit samples was reported with increase in concentration range of 200–1000 µg/ml (w/v) In NORS assay, IC_{50} value of all selected fruits was observed in the concentration range of 200-400µg/ml except with apricot fruit where IC_{50} value was reported in 400-600µg/ml.

3. Storage stability of probiotic antioxidant rich fruit supplemented probiotic buttermilk (lassi)

The pH of the all buttermilk samples prepared using free, alginate and carrageenan encapsulated *L. rhamnosus* decreased with increase in acidity up to 15 days of storage which is attributed to acidity of fruits and the acidic nature of buttermilk (Table 1). Probiotic bacteria must arrive in intestine alive and in adequate number i.e. 6-7 log CFU/g of product to confer health benefit. The probiotic buttermilk samples without fruits prepared using free probiotic culture retained probiotic values of 6.52±0.04 log CFU/ml only on day 1 of storage at 4°C and after this the probiotic count decreased continuously during storage. However the alginate and carrageenan encapsulated probiotic culture retained probiotic value of 6.24±0.06 log CFU/ml and 6.42±0.04 log CFU/ml respectively up to 5 days of storage and thereafter probiotic count decreased during storage (Table 1). However, no growth was observed on day 15 in all probiotic buttermilk products prepared using free, alginate and carrageenan encapsulated probiotic culture. The jamun, apricot, raspberries and plum supplemented lassi prepared using free probiotic culture retained probiotic values of 6.64±0.03, 6.57±0.04, 7.26±0.08 and 6.63±0.02 log CFU/ml respectively on day 1 during storage at 4°C and decreased up to day 5. No growth was observed on day 10 and 15 in lassi prepared using free probiotic culture (Table 1).

Buttermilk (lassi) samples prepared using alginate microencapsulated culture retained probiotic values of 6.42±0.03 and 6.55±0.03 log CFU/ml respectively in apricot and raspberries supplemented lassi on day 5 of storage at 4°C whereas, jamun and plum supplemented lassi retained probiotic value of 6.6±0.01 log CFU/ml only on day 1 of storage. No growth was observed on day 15 in apricot, jamun and raspberries supplemented lassi and on day 10 of storage in plum supplemented probiotic lassi. All buttermilk samples i.e. jamun, apricot, raspberries and plum supplemented prepared using carrageenan encapsulated probiotic culture retained probiotic values of 7.31±0.05, 7.15±0.06, 6.25±0.05 and 6.16±0.06 log CFU/ml respectively on day 1 of storage at 4°C and after this the count started decreasing. In plum and raspberries supplemented lassi no growth was observed on and after 10 days of storage. On the other hand, in jamun and apricot supplemented lassi no growth was observed on day 15 during storage (Table 1). In this study, a significant reduction in the number of viable probiotic cells was reported in which is attributed to the

processing conditions especially low pH and high acidity during storage.

The results obtained in the present study were compared in light with the existing literature. Hussain *et al.* [33] developed *Aloe barbadensis* Miller supplemented probiotic *lassi* (APL) and reported the decrease in pH during storage at 5°C. The probiotic cells count also decreased in this study from 8.4 log CFU/ml on day 1 to 8.0 log CFU/ml on 12th day of storage. Shukla *et al.* [34] prepared probiotic beverage from whey and pineapple juice and reported decrease in pH and increase in the titratable acidity in both whey and whey-pineapple juice blend. The reduction in the viable probiotic cells count was reported but the viability count of probiotic beverage did not fall below 10^6 CFU/ml. Mohan *et al.* [35] developed probiotic fruit juices by using *Lactobacillus acidophilus.* They reported decrease in pH and increase in acidity. The encapsulated probiotic culture was more stable as compared to free probiotic culture during storage.

Fruit supplemented lassi (4% v/v) were used for the antioxidant analysis in the present study and the results are shown in Figure 1. The significant decrease in the antioxidant activity of the fruit supplemented probiotic buttermilk was reported during storage. In DPPH radical scavenging assay the percentage scavenging decreased from 64.22±0.86 to 38.93±1.59 during storage. In FRAP assay the optical density decreased from 0.657±0.02 to 0.423±0.01 during storage up to 15 days. In NORS assay the percentage scavenging decreased from 65.60±1.62 to 39.29±3.29 during storage (Figure 1). The antioxidant activity of the fruits supplemented lassi samples also decreased over a storage period from 1-15 days, which may be attributed to denaturation during storage time as observed earlier with fruit supplemented probiotic yogurt [13]. Wicklund *et al.* [36] observed that, jelly stored at 4ºC had a higher content of anthocyanins and total antioxidant capacity than the samples stored at 20ºC, whereas there was no significant differences during dark and light storage. Lawin and Kongbangkerd [37] reported decrease in antioxidant activity of yogurts fortified with roselle syrup during storage due to loss of anthocyanin activity. El-Said *et al.* [38] developed stirred yogurt fortified with pomegranate peel extracts and evaluated the antioxidant activity of the yogurts and observed similar findings.

CONCLUSION

In conclusion different types of probiotic buttermilk (with and without fruit supplements) were prepared using free, alginate and carrageenan encapsulated

probiotic *L. rhamnosus* culture. The viable probiotic cell count reduced during storage. However, the microencapsulated probiotic culture was more stable as compared to free probiotic culture. Out of two matrix used for microencapsulation of *L. rhamnosus*, the alginate encapsulated probiotic culture was found more stable. The antioxidant activity of the fruit supplemented products decreased during storage but retained useful amount of antioxidants during storage. Based on the results of present study the apricot and raspberries fortified probiotic buttermilk prepared using alginate encapsulated and probiotic buttermilk prepared without fruits using alginate and carrageenan were found to be the most stable products. In future, the *in vivo* animal experiments or cell line studies and sensory studies will be required to prove the potentiality and acceptability of the products. The wild fruits used in the present study are still underutilized in the state and can be used for the development of fruit fortified probiotic products for daily healthy dietary consumption.

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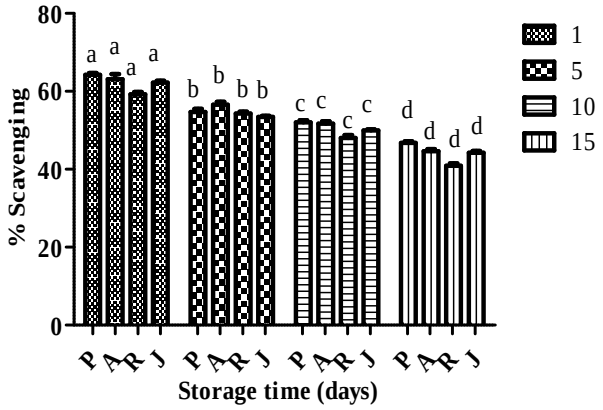
Table 1: Physicochemical and microbiological analysis of different types of buttermilk (lassi) samples prepared using free, alginate and carrageenan encapsulated probiotic *Lactobacillus rhamnosus* culture during storage at 4ºC

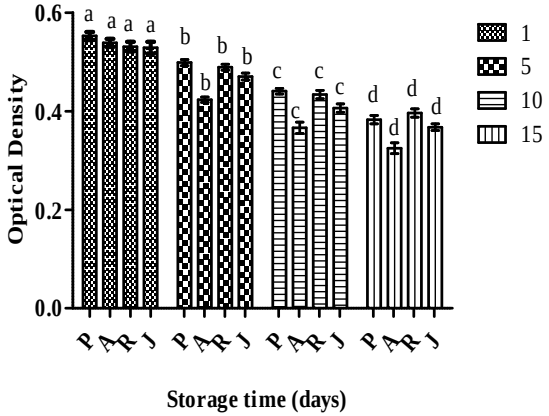
Lassi types	Storage time (days)	Lassi prepared using free probiotic culture			Lassi prepared using alginate (2 %) encapsulated probiotic culture			Lassi prepared using carrageenan (2 %) encapsulated probiotic culture		
		pH	Acidity (%)	log CFU/ml	pH	Acidity (%)	log CFU/ml	pH	Acidity (%)	log CFU/ml
Control without fruit fortification	1	3.82 ± 0.03 a	0.59 ± 0.01 a	6.52 ± 0.04 a	3.91 ± 0.02 a	0.56 ± 0.01 a	7.32 ± 0.06 a	3.92 ± 0.05 a	0.56 ± 0.02 a	7.21 ± 0.07 a
	5	3.34 ± 0.05 b	0.66 ± 0.01 a	5.59 ± 0.02 b	3.50 ± 0.04 b	0.63 ± 0 a	6.24 ± 0.06 b	3.53 ± 0.03 b	0.63 ± 0.01 a	6.42 ± 0.04 b
	10	2.71 ± 0.05 c	0.71 ± 0.01 b	2.35 ± 0.04 c	3.17 ± 0.07 c	0.71 ± 0.01 b	3.55± 0.03 c	3.00 ± 0 c	0.73 ± 0.01 b	2.61 ± 0.03 c
	15	2.46 ± 0.04 d	0.77 ± 0 c	ND	2.56 ± 0.06 d	0.77 ± 0.02 c	ND	2.80 ± 0.05 d	0.76 ± 0.01 c	ND
Jamun fortified	1	3.75 ± 0.04 a	0.60 ± 0.01 a	6.64 ± 0.03 a	3.74 ± 0.05 a	0.60 ± 0.01 a	6.6 ± 0.01 a	3.68 ± 0.04 a	0.60 ± 0.01 a	7.31 ± 0.05 a
	5	3.22 ± 0.03 b	0.71 ± 0.01 b	4.36 ± 0.05 b	3.34 ± 0.05 b	0.70 ± 0.01 b	4.57 ± 0.03 b	3.25 ± 0.15 b	0.70 ± 0.02 a	4.46 ± 0.03
	10	2.76 ± 0.04 c	0.76 ± 0 c	ND	2.85 ± 0.04 c	0.73 ± 0.01 c	2.32 ± 0.02 c	2.74 ± 0.08 c	0.76 ± 0.01 b	2.53 ± 0.04
	15	2.49 ± 0.07 d	0.81 ± 0.02 d	ND	2.44 ± 0.06 d	0.83 ± 0.02 d	ND	2.48 ± 0.06 d	0.82 ± 0.02 c	ND
Apricot fortified	1	3.59 ± 0.04 a	0.61 ± 0.01 a	6.57 ± 0.04 a	3.68 ± 0.03 a	0.59 ± 0.01 a	7.23 ± 0.05 a	3.67 ± 0.06 a	0.60 ± 0.02 a	7.15 ± 0.06 a
	5	3.33 ± 0.04 b	0.70 ± 0 b	4.44 ± 0.05 b	3.26 ± 0.10 b	0.70 ± 0.01 b	6.42 ± 0.03 b	3.25 ± 0.05 b	0.68 ± 0.01 a	5.46 ± 0.03 b
	10	2.82 ± 0.03 c	0.74 ± 0.01 c	ND	2.90 ± 0.04 c	0.75 ± 0.02 c	3.27 ± 0.08 c	2.85 ± 0.07 c	0.75 ± 0.02 b	2.50 ± 0.02 c
	15	2.52 ± 0.06 d	0.81 ± 0.01 d	ND	2.53 ± 0.02 d	0.81 ± 0 d	ND	2.46 ± 0.05 d	0.84 ± 0.02 c	ND
Raspberries fortified	1	3.53 ± 0.06 a	0.62 ± 0.01 a	7.26 ± 0.08 a	3.66 ± 0.03 a	0.59 ± 0.01 a	7.25 ± 0.05 a	3.63 ± 0.06 a	0.62 ± 0.01 a	6.25 ± 0.05 a
	5	2.97 ± 0.05 b	0.72 ± 0.02 b	5.47 ± 0.04 b	3.21 ± 0.03 b	0.70 ± 0.01 b	6.55 ± 0.03 b	3.22 ± 0.04 b	0.68 ± 0.01 a	3.33 ± 0.02 b
	10	2.70 ± 0.05 c	0.76 ± 0.01 c	ND	2.80 ± 0.05 c	0.76 ± 0.02 c	3.35 ± 0.05 c	2.91 ± 0.05 c	0.73 ± 0.01 b	ND
	15	2.40 ± 0.05 d	0.84 ± 0.01 d	ND	2.45 ± 0.05 d	0.82 ± 0.02 d	ND	2.51 ± 0.1 d	0.82 ± 0.02 c	ND
Plum fortified	1	3.54 ± 0.05 a	0.62 ± 0.02 a	6.63 ± 0.02 a	3.79 ± 0.04 a	0.58 ± 0.01 a	6.6 ± 0.01 a	3.79 ± 0.04 a	0.58 ± 0.01 a	6.16 ± 0.06 a
	5	3.23 ± 0.07 b	0.69 ± 0.01 a	4.51 ± 0.02 b	3.36 ± 0.05 b	0.69 ± 0.01 a	5.27 ± 0.04 b	3.36 ± 0.05 b	0.69 ± 0.01 b	4.6 ± 0.01 b
	10	2.80 ± 0.09 c	0.75 ± 0.02 b	ND	2.92 ± 0.05 c	0.73 ± 0.01 b	ND	2.92 ± 0.05 c	0.73 ± 0.01 c	ND
	15	2.49 ± 0.08 d	0.83 ± 0.03 c	ND	2.51 ± 0.02 d	0.81 ± 0 c	ND	2.51 ± 0.02 d	0.81 ± 0 d	ND

ND= Not determined

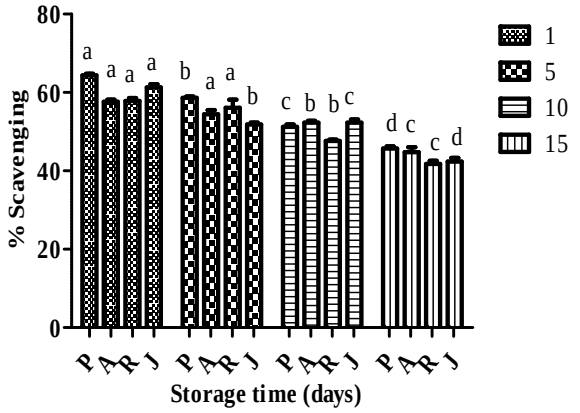
When mean values were significantly different (P < 0.05), different letters were used (a, b, c, d)



DPPH

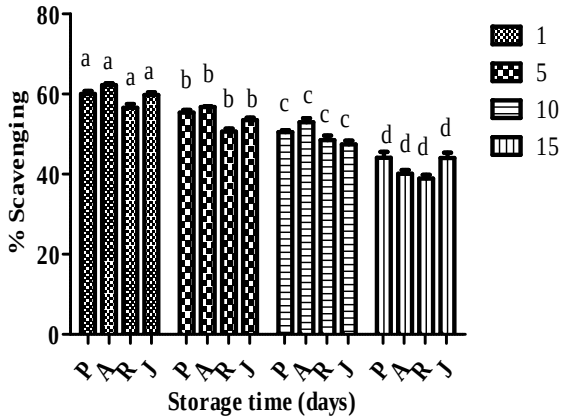


FRAP



NORS

(1)



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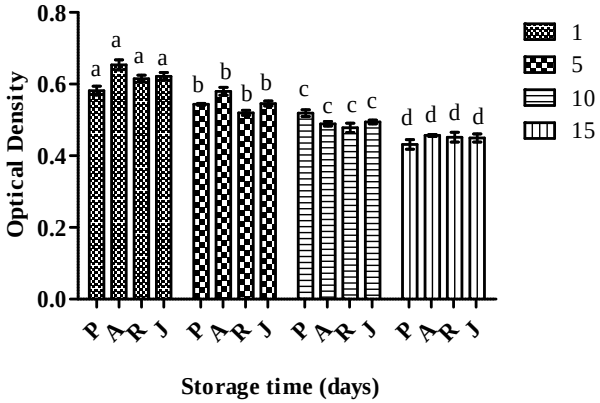
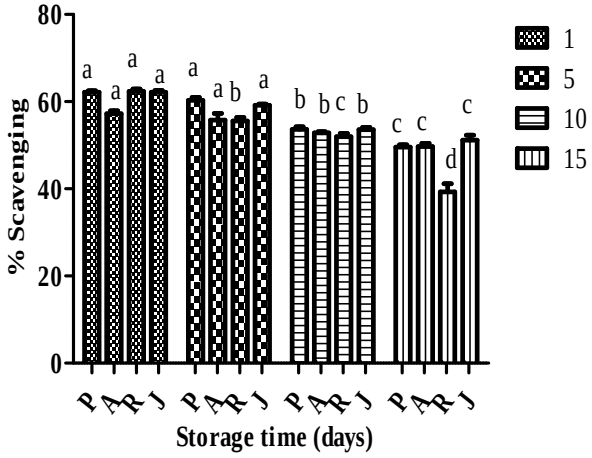
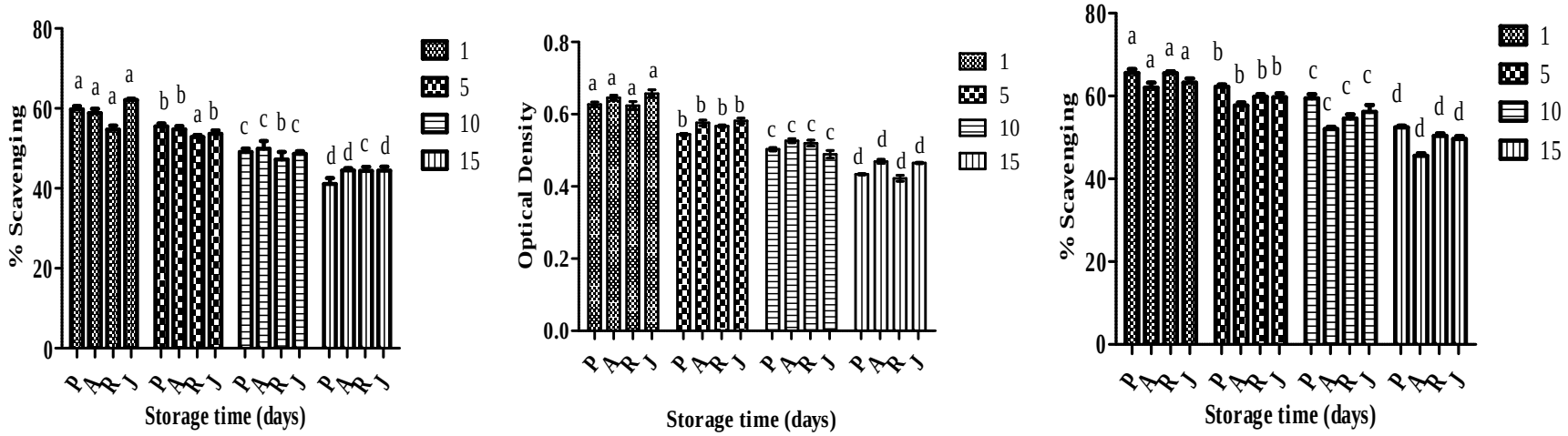


FRAP



NORS

(2)



DPPH FRAP NORS

(3)

Figure 1: Antioxidant (DPPH, FRAP, NORS) analysis of fruits fortified probiotic buttermilk developed using 1) free *L. rhamnosus* culture, 2) alginate encapsulated probiotic culture, 3) and carrageenan encapsulated culture on 1, 5, 10 and 15 days of storage at 4ºC. P- Plum, A-Apricot, R-Raspberries and J-Jamun. When mean values were significantly different (P < 0.05), different letters were used (a, b, c, d)