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Synergistic effect of some probiotics and natural dye on the improvement of yogurt properties

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Abstract Probiotics play an important role to improve yogurt characteristics and increase its health benefits properties. The effects of adding micro-encapsulated *Lactobacillus* sp. and *Streptococcus* to yogurt in addition to natural dye obtained from *(Morus rubra*) were investigated. The micro-encapsulated *Lactobacillus* sp. and *Streptococcus* sp. were added to yogurt and natural dye to determine antioxidant activity, acidity, protein content and viscosity. The results showed that the use of micro-encapsulated *Lactobacillus casei*LC12**,** *Lactobacillus acidophilus* LA5 and *Lactobacillus rhamnosus* LR22 remarkably maintained and increased the antioxidant activity as well as they decreased the undesired changes in yogurt properties involving acidity, protein content, syneresis and viscosity compared to the use of non-encapsulated strains. The data indicated that the use of *Streptococcus* improved yogurt characteristics which significantly lower than *Lactobacillus* sp. Finally, the addition of probiotics were accompanied by natural dye to yogurt remarkably increasing the beneficial healthy properties on antioxidant, acidity, protein content and viscosity. It is indicated that the priority using *Lactobacillus* sp. was better than *Streptococcus* sp.

Keywords: Probiotics, Natural dye, *Lactobacillus*, *Streptococcus*, Encapsulation, Yogurt

Introduction

Different microbial species are considered to be probiotics. They inhibit the growth of some pathogenic organisms in small and large intestine by the production of some organic acids and some active bacteriocins (Martini *et al*., 1991). Probiotic inyogurt is controlled by the National Yogurt Association rules which defines the active culture. Yogurt product should contain live (LAB)> 10^8 cells/g at the ending time of the manufacture. The mainrole of probiotic is to enhance gastrointestinal function through increasing minerals absorption as well as reduction in lactose in tolerance (Mena-Calas, 2013).

Dairy products such as milk, cheese and yogurt have been recognized as excellent sources of vitamins including riboflavin and mineral phosphorus as well ascalcium.The growing popularity of yogurt

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over the last decades has largely been increased due to its perceived health benefits. Yogurt is a coagulated milk product whichobtained by lactic acid fermentation using a specific bacteria (Naderi *et al*., 2004). Yogurt is one of the bestknown foods thatcontain many living microorganismby ingestion in sufficient amount, exerts beneficial effects to human being (Suh *et al.*, 2003).

Anthocyanins is the main component of Morus pigment that has strongly expressed oxidation resistance, antimicrobial properties and provide UV protection. Anthocyanins can also inhibit the growth of cancer cells, inflammation and anti-obesity effects. In spite of the great application of mulberry fruits into food, pharmaceutical and cosmetic industries. It is recently found that the mechanism of action related to antioxidant activity. Mulberry contains soluble plant chemicals known as bioflavonoids which are powerful antioxidants to be responsible for medicinal properties (Suh *et al.*, 2003). Anthocyanin is the major pigment responsible for color in mulberry fruits, and the major compounds were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside (Tsai *et al*., 2004; Darias Martin *et al.*, 2003). Chemical structure of anthocyanin dye was extracted from Mulberry (*Morus rubra*) plant (Figure 1)**.**

The mulberry fruits contain various polyphenols with physiological functions that offer protection tohuman (Yoo and Kim, 2016). Anthocyanins and flavonols are two majorpolyphenolic compounds in mulberry fruits. The mainflavonols in mulberry fruits include quercetin, morin andmyricetin (Dave and Shan 1996; Gustaw *et al*., 2006). The main anthocyanins include cyaniding 3-glycoside and pelargonidin 3-glycoside which are responsible for the colouring of fruits and fruit products (Gustaw *et al.*, 2009).The research finding showed that anthocyanins have strong oxidation resistance, antimicrobial properties(Barbano *et al*., 1991; Amatayakul *et al*., 2006) and UV protection (Zainoldin and Baba, 2009).

The aim was to investigate the effect of probiotic in combination with natural dye for increasing the healthy properties of yogurt.

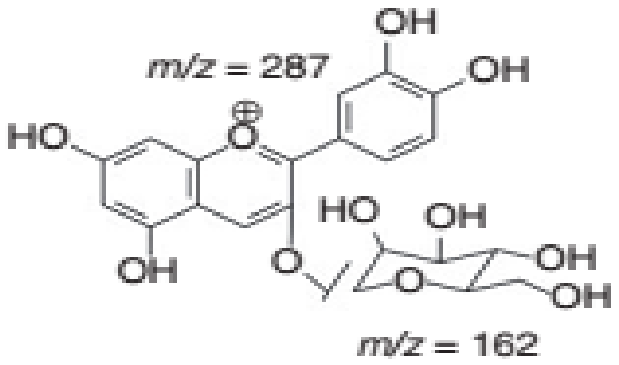

Figure 1. Chemical structure of anthocyanin dyes extracted from Mulberry (*Morus rubra)* plant

Materials and methods

Microorganisms

The microorganisms used in the current work were three strains of *Lactobacillus* sp. (*L. casei* LC12, *L. acidophilus* LA5 and *L. rhamnosus* LR22) and two strains of *Streptococcus* spp. These bacterial isolates were obtained from the Natural and Microbial Products Chemistry Department, National Research Centre. The encapsulated-*Lactobacillus* sp. and *Streptococcus* sp. were obtained from theInstitute Rosellallemand, Inc., Monteral, Quebec, Canada.

Chemicals

All chemicals used in the current work were obtained from MerckMillipore (Billerica, MA, USA) and Sigma-Aldrich (St. Louis, MO, USA). All solvents were HPLC-grade and obtained from Sigma-Aldrich.

Extraction of mulberry dye

Mulberry (*Morus rubra*)belongs to Moraceae is plant native in Asia and widely cultivated in Southern Europe for centuries because of its wide usage for many purposes(Daria- Martin *et al.*, 2003). Black mulberry (*Morus nigra*) is one of the most important species of *Morus*. The members of this genus can begrown without protection in many countries (Elmaci and Tomris, 2002).

The fresh mulberry fruits were crushed and mixed with ethanol at liquor ratio of 1:2. Extraction was carried out at a temperature of 50 ^{0}C for 2 h and followed by filtration. The filtrate was evaporated until dryness and gave crystals. A known weight was added to yogurt samples (Yoo and Kim, 2016). The fresh mulberry fruits were crushed with a mortar and mixed with solvent (methanol/TFA/H2O 80:0.5:19.5 v/v/v), keeping a material-to-liquor ratio of 1:2. Extraction was carried out at a temperature of 50 ℃ for 2 h, followed by filtration. The filtrate was diluted with distilled water (1:4 v/v) and later used for dyeing.

Maintenance of the microorganisms

Lactobacilli were maintained using MRS agar which prepared according to the manufacturer's instructions (Difco, MD, USA) where 55 g of MRS powder was weighted and suspended in 1 L of distilled water, 20 g of agar was added and the mixture was autoclaved at 121°C for 15 minutes.

Streptococcus spp. was maintained according to previously described methods Dave and Shah (1996). The following medium consists

of sucrose10 g/L, yeast extract 5 g/L, tryptone 10 g/L and $K_2HPO_4$2 g/L and agar 20 g/L which were dissolved in distilled water. The pH of mixture was adjusted to 6.8 ± 0.1 with 1 N HCl and sterilized.

Yogurt production

Skim milk powder was reconstituted at 30 ℃ and moderately mixed using a magnetic stirrer. The dispersions were refrigerated at 4 ℃ for 24 h to ensure full hydration of the powders. Yogurt samples were prepared using 1% skim milk, and placed in glass jars and heated at 85 ℃ for 30 min (Dave and Shah, 2016; Gustaw *et al*., 2006). Samples were cooled to the incubation temperature (40-42 ℃), subsequently transferred with the appropriate bacterial culture (0.1 g/L) at 40 ℃, and fermented until pH of sample reached to 4.7. After incubation, yogurt samples were stored at 4 ℃ for two weeks. The samples were examined daily to determine the grow and the yogurt properties.

Encapsulated *Lactobacillus* spp. were individually incorporated into the yogurt and mixed at the rate of 0.1 g/L. Inoculated yogurt mixture were poured into 50 mL containers and incubated at 40 ℃ and pH 4.7 was reached, then samples were cooled and stored at 4 ℃ until analysis. Yogurt production was performed in triplicates.

Enumerations

Probiotics were enumerated as previously reported by Yoo and Kim (2016) but with modifications. The appropriate amount of distilled water was added to 500 mL of MRS base medium without dextrose. The base medium was prepared by weighing the appropriate proportion of 10 g of peptone 3 g, beef extract 10 g, yeast extract 5 g, tween 80 1 g, ammonium citrate 2 g, sodium acetate anhydrous 5 g, magnesium sulphate anhydrous 0.1 g, manganese sulphate monohydrate 0.05 g, dipotassium phosphate 2 g, and agar 15 g , and diluted these ingredients in 1 L (Gustaw *et al*., 2009; Barbano *et al*., 1991).The mixture was heated to boiling with agitation before autoclaving at 121 ℃ for 15 min. Sorbitol solution (10% w/v) was prepared and filter-sterilized. The appropriate dilution of yogurt was made with 99 mL of sterilized peptone in dilution bottles. The pour plate method was performed with MRS-sorbitol agar. Petri dishes were placed in BBL Gas Paks and incubated under anaerobic condition at 37 ℃ for 72 h. A colony counter was used to assist in enumerating the colonies.

PH determination

The pH of yogurt samples was measured according to previously described methods by Gustaw *et al*. (2009) using a digital pH-meter. pH

value of yogurt samples was determined using the Oysters Series pH meter (Extech Instruments, Waltham, MA, USA). The instrument was calibrated using commercial pH 4.00 and 7.00 buffers (Fisher Scientific, Hampton, NH, USA). The instrument temperature was adjusted to the sample temperature of 8℃ ± 2 before sample pH measurement and measurements were taken in duplicates.

Protein determination

Procedure for determining milk protein content is approved by the Market Administrator Milk Test Procedures Committee and involved for determining the nitrogen content by Kjeldahl methods (Amatayakul *et al.*, 2006).

Syneresis

It was determined using a previously described method (Zainoldin and Baba, 2009) with slightly modifications. Yogurt mixture of 300 mL was poured into plastic cups. The cups of yogurt set were kept at an angle of 45 ° and spontaneous whey which collected on one side of the cup with a pipette. The amount of whey in 1 mL was measured at 22℃. The yogurt gel was allowed to stand for 1 minute and further analysis was undergone.

Apparent viscosity

Apparent viscosities were measured using a Brookfield DV-II+ viscometer (Brookfield Engineering Lab Inc., Stoughton, MA, USA) with a helipath stand at 10℃ ± 2 according to previously described by Shah (2007).

Antioxidant properties

The antioxidant activity of prepared yogurt samples containing probiotics and dye was estimated using methanolic 2, 2,-diphenyhydrazyl (DDPH)solution (Aldrich D913-2, Germany).The absorption levels of each sample was determined at 517 nm according to a previously described method (Samona and Robinson, 1994). The antioxidant property was also measured to prepare yogurt samples containingnatural dye extracted from Mulberry (*Morus rubra*) plant.

DPPH radical scavenging ability

The scavenging activity for DPPH free radical was measured according to (Zhaom, 2006) with some modifications. Six samples were

tested for their antioxidant activity through their scavenging activity against DPPH free radical. All the samples showed weak scavenging activity compared to the referenced ascorbic acid, where the highest scavenging percentage was 32.58±0.01 which obtained dye at concentration 1mg/ml. 100µl samples (1mg/ml) were mixed with 900µl of 0.1mM DPPH solution in methanol. The mixture was shaken vigorously and allowed to reach a steady state for 30 min in dark at temperature 37^0C. Decolorization of DPPH was determined by measuring the absorbance at 517 nm, and the DPPH radical scavenging was calculated according to the following equation:

$$(\%) \text{ scavenging rate} = (A_1\text{-}A_2/A_1) \times 100$$

Where $\mathbf{A_1}$ was the absorbance of the DPPH solution without sample, and $\mathbf{A_2}$ was the absorbance of DPPH with the sample. Ascorbic acid was taken as the standard. All the tests were performed in triplicates. Six samples were tested for their antioxidant activity through their scavenging activity against DPPH free radical. All the samples showed weak scavenging activity compared to the reference ascorbic acid, where the highest scavenging percentage was 32.58±0.01 which obtained dye at concentration 1mg/ml.

Results

Probiotic enumerations

The number of bacterial cells in yogurt samples showed that application of the microencapsulated *Lactobacilli* and *Streptococci* remarkably enhanced the survival of the probiotics used, since the concentrations of the used bacteria remained higher than those of non-encapsulated bacteria until the end of the experiment (Figure 2).

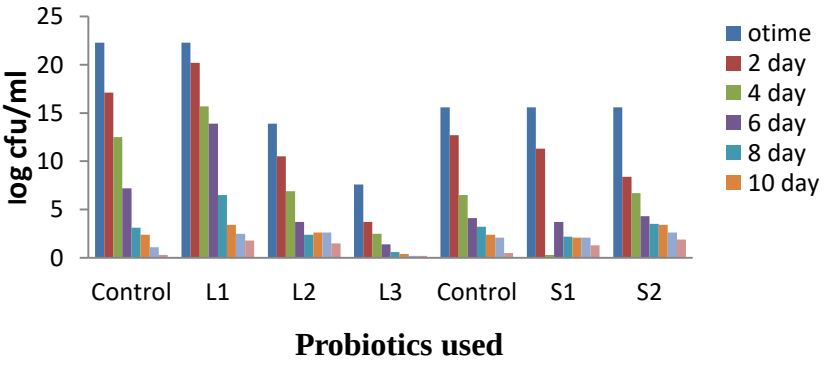

Figure 2. Enumeration of the selected probiotics; Control (The bacterial strains used without microencapsulation.), L1 (*L. casei* LC12), L2 (*L. acidophilus* LA5), L3 (*L. rhamnosus* LR22), S1 (*S. thermophilus*) and S2 (*Streptococcous* sp.)

Changes in acidity

There mark able changes of acidity during the storage time, and these changes depended on the type of bacterial strains used (Table 1).

Table 1. Acidity changes in the stored yogurt

Periods (day)	Acidity Measurement				
	L_1	**L_2**	**L_3**	**S_1**	**S_2**
0	4.4	4.4	4.4	4.4	4.4
1	4.4	4.2	4.0	4.4	4.3
2	4.3	4.1	4.3	4.3	4.2
4	4.3	4.2	4.2	4.3	4.2
6	3.5	4.5	3.7	4.3	4.1
8	3.4	4.3	3.6	4.2	4.1
10	3.3	4.3	3.5	4.2	3.6
12	3.5	4.2	3.5	4.2	3.7
14	3.5	4.1	3.5	4.2	3.6
15	3.4	4.1	3.5	4.2	3.6

L1 = *Lactobacillus casei*, L2=*Lactobacillus acidophilus*, L3=*Lactobacillus rhamnosus*, S1=*Streptococcus thermophilus*, S2=*Streptococcus* ssp.

Protein content

The effect of adding encapsulated probiotics on the protein content was determined. The results showed that the protein content of yogurt was maintained better (2933ug/ml) at the end of storage period inyogurt containing encapsulatedbacteria than the protein content of yogurts prepared with non-encapsulated strains (Figure 3).

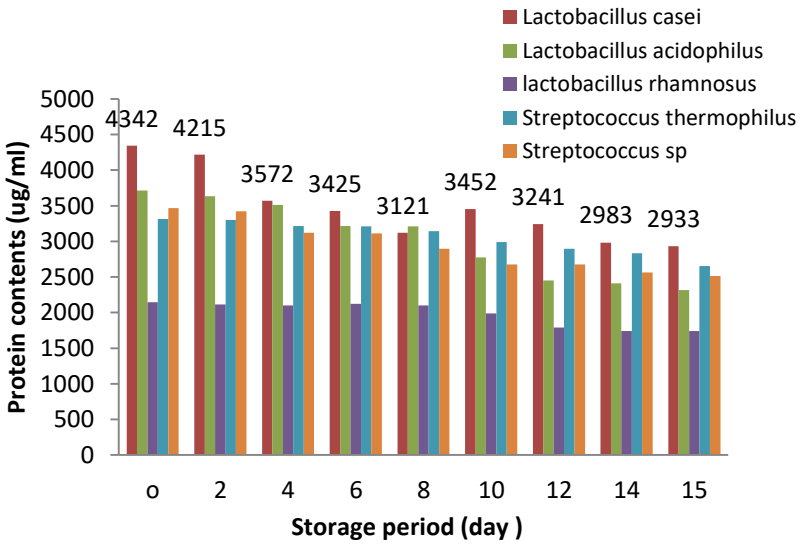

Figure 3. Effect of storage day on protein content of yogurt with probiotics

Syneresis

The spontaneous whey separation on the surface of set yogurt is defected .This problem can be reduced by increasing the milk-solid content to approximately 15% (Figure 4). The results showed that syneresiswas increased in case of yogurt containing non-capsulated probiotics compared to the capsulated one. The data revealed that the lowest syneresis was noticed by using yogurt containing microencapsulated *L. casei.*

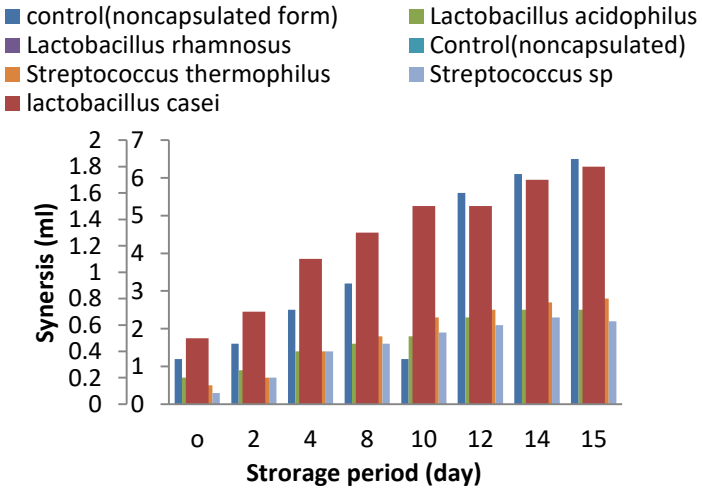

Figure 4. Estimation of syneresis of yogurt containing encapsulated probiotics

Table 2. Estimation of yogurt viscosity

	Apparent viscosity (1×10^4cP)						
Periods(day)	**Control**	**L_1**	**L_2**	**L_3**	**Control**	**S_1**	**S_2**
0	3.32	4.68	3.89	3.56	3.55	3.78	3.99
1	3.33	4.69	3.87	3.55	3.54	3.77	3.87
2	3.31	4.23	3.88	3.55	3.55	3.76	3.88
4	3.32	4.55	3.86	3.34	3.45	3.73	3.86
6	3.33	4.55	3.85	3.33	3.35	3.73	3.81
8	3.31	4.54	3.85	3.33	3.43	3.70	3.72
10	3.31	4.45	3.82	3.31	3.44	3.71	3.70
12	3.21	4.40	3.80	3.31	3.44	3.71	3.70
14	3.11	4.40	3.80	3.31	3.44	3.70	3.70

Control (the bacterial strains used without microencapsulation);
L1 *lactobacillus casei*, L2 *lactobacillus acidophilus*, L3 *lactobacillus rhamnosus,* S1 *Streptococcus thermophiles,* S2 *Streptococcus* ssp.

Viscosity

Yogurt viscosity was determined in the tested samples. The data indicated that the apparent viscosity was significantly affected by the

storage period and strain used (Table 2). The best apparentviscositywas obtained by using *L. casei* and followed by *L. acidophilus* compared to the control. On the other hand, the samples prepared with *Streptococcus* spp. showed a good viscosity but lower value than the samples prepared with *Lactobacilli*.

Antioxidant activity

The antioxidant activity of the tested samples was tested using DDPH solution. The results showed that antioxidant activity of the lactobacilli-inoculated samples was maintained during the early storage day but decreased remarkably in the subsequent days (Figure 5 and Table 3). However, the *Streptococcus* spp. had considerably lower antioxidant activity than *Lactobacilli* inoculated samples. It appeared that the antioxidant activity of yogurt supplemented with *Morous* dye showed more antioxidant properties than the other.

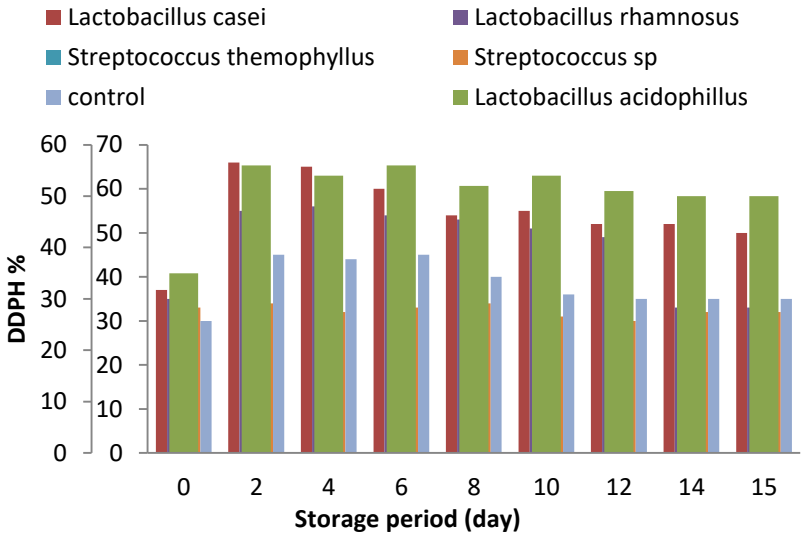

Figure 5. Effect of storage period on the Anti-oxidant activity of stored yogurt

Discussion

The number of the microoganssms in yogurt specially the probiotics varied accoding to its constituents as wellas the sourronding environment. The microencapsulated form of bacteria can overcome the severe environmental conditions. The results showed that microencapsulated bacteria had the ability against undesired changes in yogurt during storage more than the non-encapsulated one (Maganha *et al.*, 2014).

Table 3. Scavenging activity of different samples for DPPH free radical at concentration 1mg/ml (Data are presented as mean ±SD)

Strains	DPPH scavenging activity (%) With free probiotic bacteria	DPPH scavenging activity (%) of yogurt with encapsulated forms	DPPH scavenging activity (%) of yogurt with dye
Control*	1.5 ±0.01	1.5 ±0.01	1.5 ±0.01
Lactobacillus casei	12.79 ±0.04	21.79 ±0.04	21.79 ±0.04
L. acidophlous	17.75 ±0.02	37.75 ±0.02	48.75 ±0.02
L. ramnosus	12.08 ±0.1	22.08 ±0.1	32.08 ±0.3
Streptococcus thermophyllus	11.84 ±0.02	21.84 ±0.02	31.84 ±0.02
***Streptococcus* spp**	12.58 ±0.01	32.58 ±0.01	32.58 ±0.01
Ascorbic acid	97 ±1.00	97 ±1.00	97 ±1.00

*Control without probiotic bacteria

The increased in acidity of yogurt is required in the production of characteristic coagulum of yogurt, and achieved the desired texture of the product. Previous studies had also shown that enzymes in the yogurt starter bacteria converting the disaccharide lactose into lactic acid. As acid accumulates in milk and the acidity increased leading to protein denature and the milk thickens and developed an acidic taste. Variations in acidity can change the texture of yogurt. At lower acidity values, the yogurt will be sweeter and thinner, while at high acidity of yogurt it will be thicker and sour. The changed performance resulted in the growth activities of the stains which importantly in repining and manufacture of yogurt (Lieberman *et al*., 2014).

The results showed that the storage period increased syneresis in case of non-capsulated form of used bacteria. On the other hand, yogurt with higher total solid content was less susceptible to syneresis. Sodium casein is the most effective supplement that increases gel strength and reduces yogurt syneresis. Soy protein isolates have also been investigated to replace non-fat dry milk during yogurt manufacturing to improve viscosity and reduce syneresis. In higher-fat yogurts, clusters of fat globules can fill up these spaces, thus syneresis can be minimized. Previous studies have found that an increase in total solids would increase the density of yogurt matrices, thereby resulting in decreased of syneresis (Schmidt *et al*., 1995; Chen *et al*., 2003).

It was found that the addition of encaspulated form of probiotics increased the protien content in yogurt during the storage period, where the application of probiotics in yogurt was not only enhanced yogurt property but also maintained its protien cocntent during storage (Chen *et al*., 2003 and Akar *et al*., 2017). Our finding revealed the importance of *Lactobacillus casei*.

The increase of the apparent viscosity correlated with the application of an encapsulated form of probioticsis recorded by Saez-Lara *et al.* (2015). This may be due to the type of materials used in the encapsulation process

which led to high viscosity behaviour of yogurt. The results indicated that the antioxidant activity is significantly affected by the storage period as well as the type of probiotic used. Highest antioxidant activity of 69%, it was observed on the second and fourth days, and was not significantly differed on the sixth and tenth days. The lowest antioxidant activity level was about 40% infifteenth day.

DPPH scavenging assay is commonly used for screening antioxidant activity. This is due to its efficiency, simplicity and being relatively quick and inexpensive (Akar *et al.*, 2017).Antioxidant activity of a compound depends on the number of active groups, either proton or electron donating groups, and their position on the aromatic ring where ortho position is the most active due to its ability to form intra molecular hydrogen bonding followed by para position,andmeta position (Brewer, 2011; Da silva *et al*., 2017). It was found that yogurt samples containing encapsulated form of bacteria in addition to mulberry natural dye showed more antioxidant activity than without dye.

It is believed that consumption of yogurt containing LAB and other fermented probiotic-containing dairy products enhanced the gastrointestinal tract function. Many studies have indicated that there are possible health benefits of yogurt including protection against gut-associated diseases. Yogurt preparation with microencapsulated probiotics remarkably improved the yogurt properties compared to yogurt preparation with non-microencapsulated probiotics. These findings gave the benifical effect of probiotics and encourage future studies of probiotics. The antioxidant activity is significantly affected by the storage period as well as the type of probiotic. The antioxidant activity of yogurt supplemented with Morous dye showed effectively antioxidant properties.

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