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Production of functional mocha milk containing encapsulated *Lactobacillus*

rhamnosus **GG**

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ARTICLE INFO ABSTRACT

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In this study, the effect of vine training system and storage time were evaluated on some grape characteristics, such as berry contamination percentage, berry shedding percentage, berry browning rate, titratable acidity, soluble solids, total phenol and the activity of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase enzymes, in order to maintain the quality of grape during storage time. Factorial experiment was conducted based on completely randomized design with two factors of vine training system (Khazandeh, pergola and cordon) and storage time (zero, 20, 40 and 60 days after storage) with three replications during 2018-2019. After transferring the fruits to the cold storage with temperature of +4◦C, relative humidity of 85-90% and storage for two months, some characteristics of grape were examined on different days after storage. The results showed that the level of contamination of the berry increased over time, and during the storage period, the lowest level of contamination (13.706%) was related to the cordon training system. Also, after 60 days of storage, the lowest percentage of berry drop (27.535%) was observed in the fruits harvested from the cordon training system. The amount of berry browning after 60 days of storage was significantly higher than other times. The highest amount of TSS (26.182 degrees Brix) was related to the Khazandeh training system in 40 days after storage, however, a significant decrease in the value of this index was observed during 60 days after storage. The results showed that the amount of phenol increased until the end of the storage period, and the maximum amount of phenol in all three training systems was significantly higher than other days in 60 days after storage. The level of peroxidase enzyme activity in the Khazandeh training system reached its highest level at 20 days after storage, while in the other two training systems, it was significantly higher than the other days at 40 days after storage. Also, the activity of polyphenol oxidase enzyme reached its maximum level in 40 days after storage in Khazandeh and pergola training systems and then decreased. While in cordon training system, the highest activity of this enzyme was observed after 60 days of storage. The activity of phenylalanine ammonia-lyase enzyme increased during the storage period, and 60 days after storage, the highest activity of this enzyme was related to the pergola training system. In general, the results showed that the cordon training system had the best effect in maintaining the characteristics of grape during the storage period compared to other training methods.

1. Introduction

Flavored milks are ready-to-drink products made from unfermented milks of various fat contents, mixed with ingredients such as sugar or other sweeteners, cocoa powder, fruit concentrate, coffee, flavorings and other ingredients and additives. Flavored milks satisfy consumers' desire for variety and various experiences in taste, because some consumers do not like the taste of natural milk instead of flavored milk. In addition, flavored milk can encourage children to consume further milk [1]. Therefore, various flavored milks are produced in industrial scale; of which, cocoa milk, coffee milk, honey milk, soymilk and date milk can be addressed. Importance of adding various compounds to milk increases because in addition to creating special colors, aromas and tastes in products, this can increases functional effects of the milk [2]. In addition to their nutritional values, functional products include health-giving effects for the consumer and can help maintain their physical, mental and psychological conditions [3]. The major purpose of producing functional products is to use beneficial microorganisms and compounds by the consumers on a daily basis. Moreover, dairy industries use probiotics as tools for developing novel functional products [4]. From the types of functional products, products containing probiotic microorganisms can be highlighted [5]. Probiotic products should contain at least 7 log CFU/ml live probiotic cells at the time of consumption [6].

Lactobacillus and *Bifidobacteria* species are the most well-known probiotic microorganisms [8]. Naturally, *L. rhamnosus* can survive in the human intestine with health effects. The bacteria are resistant to bile and survive while passing through the human digestive system. In contrast, the bacteria do not react to immune responses. Furthermore, *L. rhamnosus* can be used in a variety of commercial probiotic products due to the bacterial resistance to acid and bile and ability to adhere to the intestinal epithelial layers [9]. After consumption, a sufficient quantity of probiotic cells must survive in the gastrointestinal tract (GIT), transporting to the host intestine. Therefore, viability of the probiotics in the products should be improved. One of the methods to preserve probiotics against adverse environmental conditions includes encapsulation of the bacteria. Encapsulation includes packing of bioactive substances inside a cover that can release their contents at a controlled rate under certain conditions. Encapsulation can help microorganisms protect themselves against adverse environments and preserve their viability and stability [10]. Cocoa includes natural antioxidants, polyphenols and essential minerals. As a result, cocoa milk can be an appropriate alternative to regular milks for adding probiotics, improving general health of various populations, especially children [11]. Coffee contains useful compounds such as caffeine and chlorogenic acid. Caffeine acts as an antioxidant and can improve functionality of the consumer's immune system. Recently, industrials have been interested in increasing functional values of the food products containing coffee, one of which includes addition of probiotics to food products, which can potentially improve overall health of the consumers [12]. Since mocha milk contains large quantities of useful antioxidants as well as calcium, addition of probiotic bacteria in such a product helps improve health of the people [11– 12]. Therefore, the major aim of this study was to investigate possibility of producing probiotic mocha milk and assessing viability of *L. rhamnosus* GG encapsulated using three carriers of sodium alginate, sodium alginatewhey protein and sodium alginate-inulin as well as *L. rhamnosus* GG in free form. Moreover, chemical and sensory characteristics of mocha milk were assessed after storage at 4 °C for 21 d.

2-Materials and Methods

2.1. Preparation of inocula

In this study, *L. rhamnosus* GG was inoculated in MRS broth (QUELAB, Canada) and incubated at 37 °C for 24 h using incubator (Memmert, Germany). Inocula were centrifuged at 3000 g for 10 min at 4 °C (Universal 320, Germany) and washed with peptone water (0.1%) (QUELAB, Canada) [13]. Quantity of the inocula was set at 10 log CFU/ml.

2.2. Encapsulation

Extrusion technique was used for encapsulation. Briefly, 2% sodium alginate solution, 1% inulin and 8% whey protein were prepared. Then, sodium alginate and bacterial suspensions, sodium alginate-inulin and bacterial suspensions and sodium alginatewhey protein and bacterial suspensions were prepared and added dropwise to sterile calcium chloride solution (0.1 M) using syringes. The produced beads were washed with peptone water (0.1%) [13] and dried using freeze-dryer (Dena Vacuum, Iran) [7].

2.3. Preparation of probiotic mocha milk

Mocha powder was prepared using cappuccino powder, hot chocolate powder, non-dairy creamer powder, coffee powder, sugar, inulin powder and carrageenan gum. This was mixed with encapsulated and free microorganisms at a ratio of 10%, added to sterile milk and stored at 4 °C using glass bottles.

2.4. Encapsulation efficiency

Encapsulation efficiency (EE) is the survival rate of microorganisms during the encapsulation process, calculated using Eq. (1) as follows:

 $EE\% = \text{NA}/\text{NB} \times 100 \text{ Eq. (1)}$

Where, NA was the number of live cells (log CFU/ml) released from capsules and NB was the number of live free cells (log CFU/ml) used in encapsulation processes [14].

2.5. Viable cell count

Encapsulated bacteria were released using sodium citrate. Generally, 1 ml of milk was added to 9 ml of sterile sodium citrate (0.1 M) and mixed for 5 min using magnetic stirrer (PIT 300, Iran). Then, mixture was cultured on MRS agar (QUELAB, Canada) after preparing serial dilutions. Then they were incubated in anaerobic conditions at 37°C for 72 hours and counted [15].

2.6. pH analysis

Briefly, pH was assessed using digital pH meter (Metrohm 827, Switzerland) [16].

2.7. Antioxidant activity assay

Assessment of radical scavenging activity was carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Then, absorbance of the solution was measured at 517 nm using spectrophotometer (Unico UV 2100, China) [17]. Radical scavenging activity was calculated based on Eq. (2) as follows: Radical scavenging activity $\% = (Abs \text{ control} -$ Abs sample / Abs control) \times 100 Eq. (2)

2.8. Total phenolic content

Measurement of total phenol content (TPC) was carried out using Folin-Ciocalto method. Absorbance at 765 nm was measured using spectrophotometer (Unico UV 2100, China). The standard curve was prepared by increasing density of gallic acid and the phenolic compounds were reported as mg of gallic acid (GAE) per liter [16].

2.9. Sensory evaluation

In general, 40 trained general evaluators were recruited and sensory characteristics of the products were evaluated for texture desirability, taste desirability, odor desirability and overall acceptance based on 6-point hedonic test. A score of 6 was considered for the most desirable feature [4].

2.10. Statistical analysis

In this study, results were analyzed based on a completely random design in form of factorial test. Analysis of variance and comparison of means were used using LSD test at 95% confidence level and SAS software v.9.1.

3-Results and Discussion

3.1. Encapsulation efficiency

The aim of encapsulation is to create an appropriate environment for microorganisms to survive longer during storage as well as releasing in appropriate organs (e.g., the small intestine) [18]. As previously stated, EE is usually defined as "the number of probiotic microorganisms counted in the capsules, compared to the initial number used" [19]. The EE of *L. rhamnosus* GG (Fig. 1), which was encapsulated with sodium alginate, sodium alginate-whey protein and sodium alginateinulin carriers, included no statistically significant differences ($p > 0.05$). A reason for the good efficiency includes mild conditions of the encapsulation method $(25 \degree C)$ [14]. In previous studies, EE was more than 80% for *L. acidophilus* encapsulated with sodium alginatewhey protein [14] and *L. casei* encapsulated with sodium alginate-pea protein [5] and more than 90% for *L. brevis*. encapsulated with sodium alginate-soy protein isolate [20] and *L. acidophilus* encapsulated with sodium alginateinulin [13], showing that the reason included functional characteristics of the proteins and prebiotics as well as their direct effects on EE [7–14].

Figure 1. Encapsulation efficiency (%) of *Lactobacillus rhamnosus* GG in sodium alginate (EnA LR), sodium alginateinulin (EnA and I LR) and alginate-whey protein (EnA and W LR)

3.2. Viable cell count

The major value of a product containing probiotic bacteria includes the number of living cells in the product until consumption. Due to the necessity of the survival of probiotics when probiotic products are consumed, effects of encapsulation on the survival of *L. rhamnosus* GG bacteria encapsulated in mocha milk was studied in the present study. Table 1 shows the survival rates of encapsulated and free *L. rhamnosus* GG during the storage. On the first day after production, numbers of *L. rhamnosus* GG cells in all treatments included no statistically significant differences ($p > 0.05$). Viability of *L. rhamnosus* GG decreased during the storage; however, decrease rates of *L. rhamnosus* GG encapsulated in all the carriers were significantly lower than those of *L. rhamnosus* GG in free form $(p < 0.05)$, meaning that use of sodium alginate, sodium alginatewhey protein and sodium alginate-inulin as encapsulation carriers resulted in further survivals of *L. rhamnosus* GG. This verified positive effects of encapsulation on the protection of probiotics during the product storage. In other words, capsules created a

physical barrier between the microorganisms and the environment. Hence, access of microorganisms to nutrients in the environment decreased, causing less growth and activity of the microorganisms [11]. In this study, survival of encapsulated *L. rhamnosus* GG was greater than 7 log CFU/ml during storage for 21 d. Relatively, previous studies on the protective roles of capsules on the survival of *L. acidophilus* encapsulated with sodium alginatewhey protein at 9 log CFU/ml [14], *Bifidobacterium longum* encapsulated with sodium alginate-whey protein at 7 log CFU/ ml [15] and *B. lactis* encapsulated with sodium alginate-inulin at 8 log CFU/ml in goat milk [22] reported similar results.

flavored milk infused with grape juice

Table 1. Assessment of the viability of *Lactobacillus rhamnosus* GG (CFU/ml) in mocha milk

In each column, values with different lowercase letters are significantly different (p<0.05), and in each row, the values with different capital letters are significantly different (p<0.05). Mocha milk containing free form of *Lactobacillus rhamnosus* (LR), encapsulated *Lactobacillus rhamnosus* in sodium alginate (EnA LR), sodium alginate-inulin (EnA+I LR) and in sodium alginate-whey protein (EnA+W LR).

3.3. pH analysis

Based on the results from Fig. 2, pH of mocha milk containing encapsulated and free *L. rhamnosus* GG decreased during the storage; however, the pH decreases in mocha milk containing encapsulated *L. rhamnosus* GG were significantly lower than those in other samples ($p < 0.05$). A reason for this included easier access of *L. rhamnosus* GG to nutrients in the environment as well as further biological activities and production and release of organic acids by the probiotics. Previous studies showed that decreases in pH and increases in acidity in cocoa milk containing encapsulated *L. casei* and *B. animalis* were less [11], while decreases in pH and increases in acidity in

containing *Bacillus coagulans* were more possibly due to the microorganism access to grape juice as a nutrient needed for the bacterial activity and lactic acid production [23]. In mocha milk containing *L. rhamnosus* GG encapsulated with sodium alginate-inulin, pH was significantly lower than that in mocha milk containing *L. rhamnosus* GG encapsulated with sodium alginate and sodium alginate-whey protein ($p < 0.05$). This occurred possibly due to the acidic activity of inulin, when it was dissolved in milk. These findings were similar to those by Gandomi et al., who reported decreases in pH after adding inulin to apple juice and attributed it to the mild acidic behavior of inulin after dissolution [15].

Figure 2. Assessment of the pH of mocha milk containing free and encapsulated forms of *Lactobacillus rhamnosus* GG at 4 °C for 21 d

The means with different letters at the level of 5% of the LSD test are significantly different. Mocha milk containing free form of *Lactobacillus rhamnosus* (LR), encapsulated *Lactobacillus rhamnosus* in sodium alginate (EnA LR), sodium alginate-inulin (EnA+I LR) and in sodium alginate-whey protein (EnA+W LR).

3.4. Antioxidant activity assay

Cocoa and coffee include bioactive compounds with antioxidant characteristics. Cocoa powder includes averagely 75% of antioxidant characteristics [24]. Due to their metabolic activities, lactic acid bacteria (LAB) are able to produce antioxidant compounds such as phenolic compounds [25]. In this study, effects of storage time on the antioxidant capacity of mocha milk containing encapsulated and free *L. rhamnosus* GG and control were significant, increasing their antioxidant capacity $(p < 0.05)$ (Table 2). Mocha milk containing free *L. rhamnosus* GG significantly included the highest antioxidant capacity, while control mocha milk included the lowest antioxidant capacity $(p < 0.05)$ (Table 2). A possible reason included the greater metabolic activity of free *L. rhamnosus* GG, compared to the encapsulated *L. rhamnosus* GG. Madhu et al. reported increases in antioxidant capacity of probiotic yogurts containing *L. plantarum* during storage of 28 d due to the production of bioactive compounds with antioxidant characteristics [26]. Marius et al. reported the highest antioxidant capacity in fruit juices containing *L. paracasei* on the last day of storage [25]. Although probiotics include further proteolytic characteristics and growth, products produced with them include further antioxidant characteristics as well [24]. Technically, capsule created a physical barrier between *L. rhamnosus* GG and the environment, decreasing the bacterial metabolic activities [11]. Additionally, mocha milk containing *L. rhamnosus* GG encapsulated with sodium alginate-whey protein included significant increases in antioxidant capacity (*p* < 0.05). In addition to the production of bioactive compounds with antioxidant characteristics by *L. rhamnosus* GG, a good reason was linked to amino acids in whey protein, which increased the antioxidant capacity. Relatively, it could be associated to the higher antioxidant capacity in the fermented drink containing whey amino acids such as cysteine and tyrosine with alpha-lactalbumin and beta-lactoglobulin in the whey protein and whey pasteurization during processing [24].

free *L. rhamnosus* GG were significantly higher than those in mocha milk containing encapsulated *L. rhamnosus* GG and control mocha milk $(p < 0.05)$ (Table 2). This occurred possibly due to the higher metabolic activity of free *L. rhamnosus* GG with better access to environmental nutrients and metabolism of phenolic compounds [11]. Coffee and cocoa

Table 2. Antioxidant capacity (%) and total phenolic content [mg(GAE)/100 ml] in mocha milk containing encapsulated and free forms of *Lactobacillus rhamnosus*

Mocha milk	Antioxidant capacity (%)		Total phenolic content (mg(GAE) /100 ml)	
	Day 1	Day 21	Day 1	Day 21
$MM+EnA LR$	$39.90 + 4.97^b$	$57.64 + 3.92$ ^{bc}	465.13 ± 28.85 ^{de}	$703.37 \pm 32.84^{\circ}$
$MM+EnA+I LR$	$32.21 + 4.92^{\circ}$	48.08 ± 8.73 ^{cd}	416.87 ± 24.25 ^{ef}	580.27 ± 40.41 °
$MM+EnA+w LR$	$41.00 + 4.84^b$	$60.55 + 5.69^b$	471.13 ± 28.15 ^{de}	$737.47 \pm 50.85^{\rm b}$
$MM+LR$ MM	61.69 ± 3.21 ^a $29.54 + 6.53$ °	80.24 ± 3.31 ^a 42.23 ± 3.69 ^d	479.00 ± 41.62 ^d 379.97 ± 47.11 ^f	$925.93 \pm 35.58^{\text{a}}$ 512.40 ± 16.38 ^d

The means with different letters at the level of 5% of the LSD test are significantly different. Mocha milk (MM) containing free form of *Lactobacillus rhamnosus* (LR), encapsulated *Lactobacillus rhamnosus* in sodium alginate (EnA LR), sodium alginate-inulin (EnA+I LR) and in sodium alginate-whey protein (EnA+W LR).

3.5. Total phenolic content

Technically, LABs are able to produce phenolic compounds due to their metabolic activities [25]. These bacteria need lactose for their activity. Since milk contains a sufficient quantity of lactose, it is an appropriate environment for the activity of LAB and production rates of phenolic compounds increase during the storage [27]. Based on the results of Table 2, contents of phenolic compounds from all samples increased significantly during the storage ($p < 0.05$). Phenolic compounds in mocha milk containing

include several phenolic compounds such as epicatechin and catechin, increasing phenolic contents into mocha milk samples [24]. Madhu et al. investigated phenolic compounds of yogurts containing *L. fermentum* and *L. plantarum* and stated that the phenolic compounds in these yogurts were more than those in control yogurts, due to the fermentation activity of the highlighted LAB as well as production of phenolic compounds [26].

Table 3. Sensory evaluation of mocha milk containing encapsulated and free form of *Lactobacillus rhamnosus*

The means with different letters at the level of 5% of the LSD test are significantly different. Mocha milk (MM) containing free form of *Lactobacillus rhamnosus* (LR), encapsulated *Lactobacillus rhamnosus* in sodium alginate (EnA LR), sodium alginate-inulin (EnA+I LR) and in sodium alginatewhey protein (EnA+W LR).

3.6. Sensory evaluation

Mocha milk containing *L. rhamnosus* GG in encapsulated and free forms did not include statistically significant differences from the control sample for desirability of smell, taste and overall acceptance (Table 3), showing that addition of *L. rhamnosus* GG with encapsulated and free forms included no effects on these characteristics (*p* < 0.05). Assessing desirability of the texture of the samples, the control sample first received the highest score followed by mocha milk containing free *L. rhamnosus* GG. It has been reported that sizes of the capsules include effects on the appearance and texture of food products and capsules with dimensions greater than 1 mm make the products become rough and sandy [28]. Since encapsulation by extrusion method usually produces particles with large dimensions, it creates unfavorable textures of the products; thus, mocha milk containing encapsulated *L. rhamnosus* GG included less texture desirability [11]. In the present study, mocha milk containing *L. rhamnosus* GG encapsulated with sodium alginate-whey

protein received the lowest score for texture desirability.

4-Conclusion

Based on the results of this study, use of sodium alginate, sodium alginate-inulin and sodium alginate-whey protein carriers provide good encapsulation efficiency. Addition of *L. rhamnosus* GG leads to increases in nutritional and functional values of the mocha milk. Furthermore, antioxidant capacity and total phenol content of the mocha milk increase during the storage. In addition, pH changes in mocha milk containing *L. rhamnosus* GG can be delayed by encapsulation. It is possible to produce mocha milk containing encapsulated *L. rhamnosus* GG if stored at refrigerator temperatures and survival of encapsulated *L. rhamnosus* GG in mocha milk is appropriate for up to 21 d. Based on the evaluated sensory characteristics, mocha milk containing encapsulated *L. rhamnosus* GG can be marketed with the necessary modifications to improve texture of the product.

5-References

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مجله علوم و صنایع غذایی ای ران

مقاله علمی_پژوهشی

بررسی تولید شیر موکای فراسودمند حاوی باکتری الکتوباسیلوس رامنوسوس *(GG (***انکپسوله شده 2* ، مهشید جهادی ¹ هانیه نیلفروشزاده**

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