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Scientific Research

Whey permeate-based isotonic beverage formulation containing whey protein probiotic and peptides Co-encapsulated in alginate Matrix

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ABSTRACT

This study aimed to formulate a functional isotonic sports drink based on whey permeate containing probiotics and whey peptides encapsulated by alginate. The effect of hydrolyzed whey production as a natural antioxidant and whey as a coating of microcapsules in the formulated drink was investigated. Encapsulation of probiotics and whey peptides helped to improve the mechanical properties of the capsule, fermentation activity and survival of probiotics during 28 days of storage. Alginate capsules coated with whey culture showed better encapsulation efficiency, sphericity factor and antioxidant composition before and after fermentation compared to uncoated capsules. Hydrolysis had a positive effect on the antioxidant properties and viability of probiotic bacteria during 28 days of storage. Formulated whey-based sport drink can be a natural and functional alternative to existing commercial drinks.

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1- Introduction

Sports drinks have been designed to improve hydration by stimulating fluid intake, reabsorption, and fluid retention. Isotonic drinks based on whey water are proposed as an alternative hydration source for sports drinks due to their higher electrolyte concentration and similar carbohydrate content. Whey water, a byproduct of the ultrafiltration process of sweet whey, contains lactose (as the main forming element) along with several water-soluble vitamins, making it nutritionally important [1].

Fermentation is an effective method for producing hydro-electrolytes based on whey water, which can also serve as an important carrier for probiotics. Additionally, lactic acid bacteria produce various inhibitors that can increase the shelf life of fermented products [2].

Recent advancements in the production of value-added fermented products, along with increased consumer awareness about the use of natural ingredients instead of chemicals, highlight the high potential of natural substances with antioxidant activity for the production of functional beverages. These types of beverages not only have the potential to be very beneficial in terms of food safety but can also enhance the nutritional value of food products by utilizing the antioxidant benefits of these natural substances [3].

Numerous studies have been conducted regarding the health-related properties of whey protein peptides, including antioxidant properties, immune system regulation, anti-cancer effects, and increased bioavailability of minerals. Whey proteins can undergo changes during production processes such as fermentation and hydrolysis, which enhance their biological activity and antioxidant properties. In this complex process, several factors influence the production of bioactive peptides. Generally, the optimal conditions for fermentation and bacterial growth are not ideal for achieving the required degree of hydrolysis necessary for peptide production. For this reason, some manufacturers prefer to use ready-made bioactive peptides rather than producing them during the process. As a result, in some products, proteins and peptides are added during or after production to enhance their antioxidant properties [4].

Another important issue that affects probiotic products is the low viability of probiotics during storage and digestion. Encapsulation seems to be an excellent way to protect microorganisms from environmental conditions. Alginate hydrogels are widely used for cell encapsulation due to their ease of use, non-toxicity, and low cost. On the other hand, alginate beads are sensitive to acidic environments like the stomach or certain dairy products, and they are even unstable in the presence of some compounds that form chelation complexes, such as lactate and phosphate.

To address this issue, whey protein coatings can act as an effective solution by enhancing the structural integrity of alginate microcapsules and protecting them from external factors. The electrostatic interaction between whey protein and alginate helps with adhesion and stability of the coating, thus improving the performance of the encapsulation system. Furthermore, whey protein coatings not only increase the stability and viability of encapsulated materials but also serve as an additional protein source, which can improve the nutritional value of the final product.

The goal of this study was to formulate a whey protein-based hydro-electrolytic beverage with improved antioxidant capacity, longer probiotic viability, and optimized sensory properties. To achieve this goal, whey protein peptides and probiotics were co-encapsulated in alginate-based carriers, and whey protein concentrate was used to coat the microcapsules for additional protection.

2 - Materials and Methods

3) Whey Protein Isolate (WPI) powder (containing < 90% protein) was purchased from Protein Ocean Food Company. Whey water and whey protein concentrate (ultrafiltrated and concentrated product from skim cow milk by reverse osmosis) were obtained from Ramak Dairy Company in Karaj. Sodium alginate with medium viscosity (23.4.3 EC) and all solutions were purchased from Sigma-Aldrich Company.

Starter Lactobacillus plantarum (1058 PTCC) and Lactobacillus casei (1608 PTCC) were obtained from Tak Gene Biotech (Tehran, Iran), with initial cell counts of 1*10^9 CFU/ml. MRS medium and agar were prepared by Ibersco Company (Tehran, Iran).

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2-1 - Hydrolysis of Whey Protein Isolate

HWPI was prepared by dissolving WPI in a 10 mM phosphate buffer (pH 7) at a concentration of 5% (w/v). The suspension was then stirred and maintained for 30 minutes at a temperature of 37°C in the laboratory environment for hydration. The pH of the suspension was adjusted to 2.6 (for pepsin enzyme activity) using 2M HCl and NaOH. Pepsin was added to the substrate at a ratio of 1:40. After hydrolysis, the pH of the suspension was adjusted to neutral with 2M NaOH. Finally, the suspension was heated to 90°C for 15 minutes to deactivate the enzymes. The degree of hydrolysis (DH) was determined using the OPA method presented by Nielsen, Petersen, and Dambmann in 2001 [7].

2-2 - Antioxidant Capacity of WPH

The antioxidant capacity of the hydrolyzed whey protein was measured using the method presented by Lowry and colleagues in 1951 [8].

2-3 - Probiotic Inoculation

A mixture of Lactobacillus plantarum and Lactobacillus casei was inoculated in MRS broth and incubated at 37°C for 48 hours. The mixture was then centrifuged for 15 minutes and washed with a solution of 0.85% NaCl. After centrifugation and washing with phosphate buffer (10 mM, pH 7), a solution containing approximately 1.5*10^9 CFU/ml was prepared [9].

2-4 - Encapsulation of Probiotic Starter Culture

Alginate (ALG) microcapsules were obtained by the method of electrostatic extrusion. Sodium alginate solutions (1.5% w/v) were dissolved in distilled water and pasteurized in a water bath at 60°C for 60 minutes. Isolated whey protein was dissolved in deionized water (10% w/v) and magnetically stirred for 1 hour at room temperature. After adjusting the pH to 7.0, it was pasteurized at 78°C for 45 minutes to fully denature the proteins. The solutions were cooled overnight at room temperature. The probiotic culture was diluted in a solution of whey, sodium alginate, and hydrolyzed whey protein. The encapsulated bacterial cells were produced using a capsule machine (AG Inotech, Dottikon, Switzerland) equipped with an 80-micron nozzle. The device was set to a feeding rate of 5 milliliters per minute. Finally, the produced microcapsules were collected in 200 milliliters of a 0.5 mM CaCl2 solution and solidified after 30 minutes. Ultimately, the capsule-containing suspensions were collected in sterile flasks, washed, and filtered with deionized water. Finally, they were dissolved in 100 milliliters of 10 mM phosphate buffer (pH = 7) and stored at 4°C [10].

2-5 - Microcapsule Coating

The microcapsules were coated by immersion in isolated whey protein. To do this, the alginate microcapsules obtained from the CaCl2 solution were stirred for 10 minutes in a denatured WPI solution (10% w/w) and then transferred to a 0.1 mM CaCl2 solution. Finally, the microcapsules were collected and washed with deionized water.

2-6 - Encapsulation Efficiency (EE)

The amount of encapsulated probiotics during the process was calculated based on the following equation:

$$EE\% = (N / N0) \times 100$$

Where N is the number of viable cells (log CFU/g) released from the capsules and N0 is the number of viable cells (log CFU/g) in the suspension solution before the encapsulation process [11].

2-7 - Characteristics of Micro-Particles

The morphology of the micro-particles was evaluated using a light microscope (Oberkochen, Germany) [12].

The particle diameter and particle size distribution were determined using the Mastersizer 3000 (Malvern, Germany) device [13].

2-8 - Production of Fermented Beverages

Ultrafiltered whey protein concentrate was placed in 250 ml Erlenmeyer flasks. The treatments included samples inoculated with probiotics and WPH encapsulated in 1% (w/w) Alginate, probiotics and WPH encapsulated in

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Alginate and coated with WPI. All samples were incubated at 42°C. The incubation time was set between 4 to 4.5 hours to reduce the pH to 5. The products were then stored at temperatures below 5°C in a refrigerator for 28 days [14].

2-9 - Antioxidant Capacity of Whey Protein-Based Beverages

The antioxidant capacity of the samples before and after fermentation, and also during the 28-day storage (every 7 days), was determined. For this purpose, the whey-based beverage was filtered through membranes with pore sizes of 7.0 micrometers. The filtrate (substrate) was then sampled and analyzed for antioxidant capacity. The obtained solution was analyzed using the DPPH method. The free radical scavenging activity was determined using the DPPH free radical scavenging method. The samples were mixed with methanol in a 1:4 ratio and centrifuged at 6000 rpm for 10 minutes. After centrifugation, 1 ml of the supernatant was mixed with 1 ml methanol and 1 ml of the free radical DPPH solution. The mixtures were vortexed for 10 seconds to homogenize and then left in the dark for 30 minutes to react. The absorbance of the sample was determined using a spectrophotometer (NJ, SUV 2100 S, Unico) at a wavelength of 515 nm. All analyses were performed in triplicate. The percentage of DPPH radical scavenging activity was calculated as follows:

% DPPH radical scavenging activity = 100 × [Control Abs / (Control Abs - Sample Abs)]

...where Control Abs and Sample Abs refer to the absorbance values of the blank sample and the DPPH reagent solution, respectively, at a wavelength of 515 nm. Measurements were performed in triplicate [15].

2-10 - Stability of Fermented Beverages

The fermented beverages were sampled at 7-day intervals for a period of 28 days and analyzed for viable cell count, pH value, and acidity. The pH value was measured using a pH meter (WTW82362, Wellheim, Germany) at room temperature. The titratable acidity was determined by the Soxhlet-Henkel method. The number of probiotic bacterial cells was determined by a total count method on MRS agar. The cell count was expressed as mL/CFU log10 for free cells and g/CFU log10 for encapsulated cells. The encapsulated cells were released by adding 1 gram of capsules to 9 milliliters of sodium citrate (0.2% weight/volume) [16].

3 - Statistical Analysis

The experimental design was performed using a factorial experiment in a randomized complete block design with three replications. To find significant differences between the mean values obtained, ANOVA and Duncan's multiple range test were performed using SPSS software. In all statistical analyses, a significance level of ($p<0.05$) was considered.

4 - Results and Discussion

4-1 - Encapsulation Efficiency (EE%), Size, and Morphology of Microcapsules

The morphology of ALG microcapsules without coating and coated with WPI is shown in Figure 1. The microcapsules without coating were observed under optical microscope images with approximately double the size, using the nozzle diameter for feeding in these experiments. They appeared as nearly spherical microcapsules with a compact surface. The coating of ALG microcapsules with whey protein isolate added approximately 50 micrometers to their diameter (Table 1), providing a uniform coating surface. For proper protection of probiotics without affecting the sensory characteristics of food products, the microcapsules should have a diameter between 40 and 100 micrometers. However, to protect probiotics during transit through the digestive system at the pH of gastric juice, only capsules with diameters greater than 100 micrometers are effective [17].

The results related to the encapsulation efficiency of probiotics in uncoated and coated microcapsules are shown in Table 1. The results showed that coating with whey protein isolate increased the encapsulation efficiency. These results align with the study by Doherty and colleagues in 2011, which found that the whey protein coating, with its functional properties, directly impacts the encapsulation efficiency and enhances it [18].

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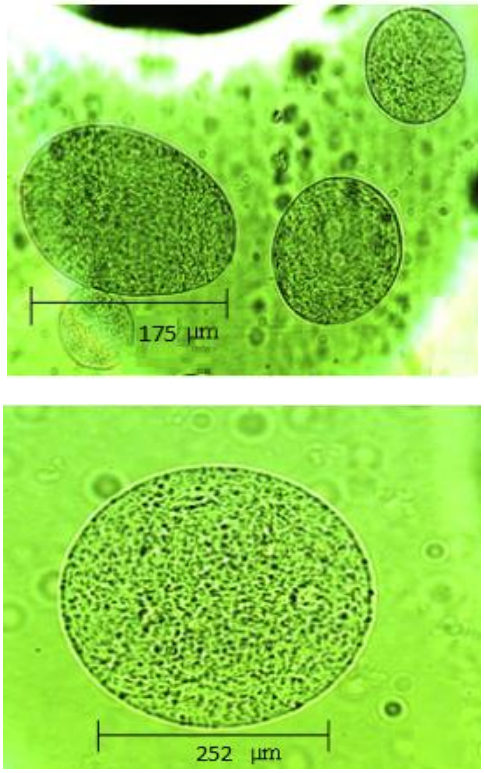
Table 1. Encapsulation Efficiency and Mean Diameter of the alginate (ALG) and WPI coated alginate (WPI/ALG) beads

Treatments	Diameter (µm)	EE (%)
ALG bead	170[a]	88.05 ± 1.05
WPI coated ALG bead	250[b]	91.85 ± 0.08

Means followed by different lowercase letters differ statistically in column *($p < 0.05$)*. The values obtained are the means ± standard deviation of triplicates.



Figue1. Encapsulated and coated probiotics under microscope

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4-2 - Antioxidant Capacity, pH, and Acidity of Fermented Beverage

The antioxidant capacity of microcapsules and whey protein-based substrates is shown in Figure 2 for samples containing uncoated ALG microcapsules and those coated with WPI. The antioxidant capacity in both coated and uncoated microcapsules decreased during fermentation, but the ALG microcapsules coated with WPI showed less of a decrease in antioxidant activity compared to the uncoated ones. This may be due to active peptides and amino acids being encapsulated and migrating from the surface of the porous microcapsules to the substrate to some extent, being used for bacterial growth inside the microcapsules during fermentation. Coating with WPI led to a more controlled release of bioactive molecules compared to the uncoated microcapsules. Additionally, the probiotic culture produces various bioactive molecules with antioxidant activity during fermentation, but the reduction in antioxidant capacity in the carriers suggests that the production of these molecules was less than the consumption of peptides with antioxidant capacity by the culture, and the migration of those peptides into the substrate.

During storage, the DPPH inhibition capacity of both coated and uncoated microcapsules increased during the first 14 days of storage, then decreased until the 28th day. The uncoated microcapsules showed a greater reduction in antioxidant capacity. This could be due to changes in the structure of the carrier, resulting from the bacterial metabolic activity and the release of material between the capsules and the substrate during storage [11]. Coating the microcapsules with WPI improved the controlled release of bioactive molecules by increasing the porosity of the capsule matrix and reducing the release rate.

The antioxidant capacity of the substrate containing ALG microcapsules during fermentation and the first 7 days of storage was significantly higher than that of the samples containing coated microcapsules. However, after 28 days of storage, the antioxidant capacity significantly decreased. This may be due to the higher release rate of bioactive peptides from the uncoated microcapsules to the substrate during fermentation and the first 7 days of storage compared to the coated microcapsules. The substrate containing coated microcapsules showed higher antioxidant capacity compared to uncoated microcapsules after 28 days of storage. This difference is due to the delayed and controlled release of bioactive peptides and amino acids.

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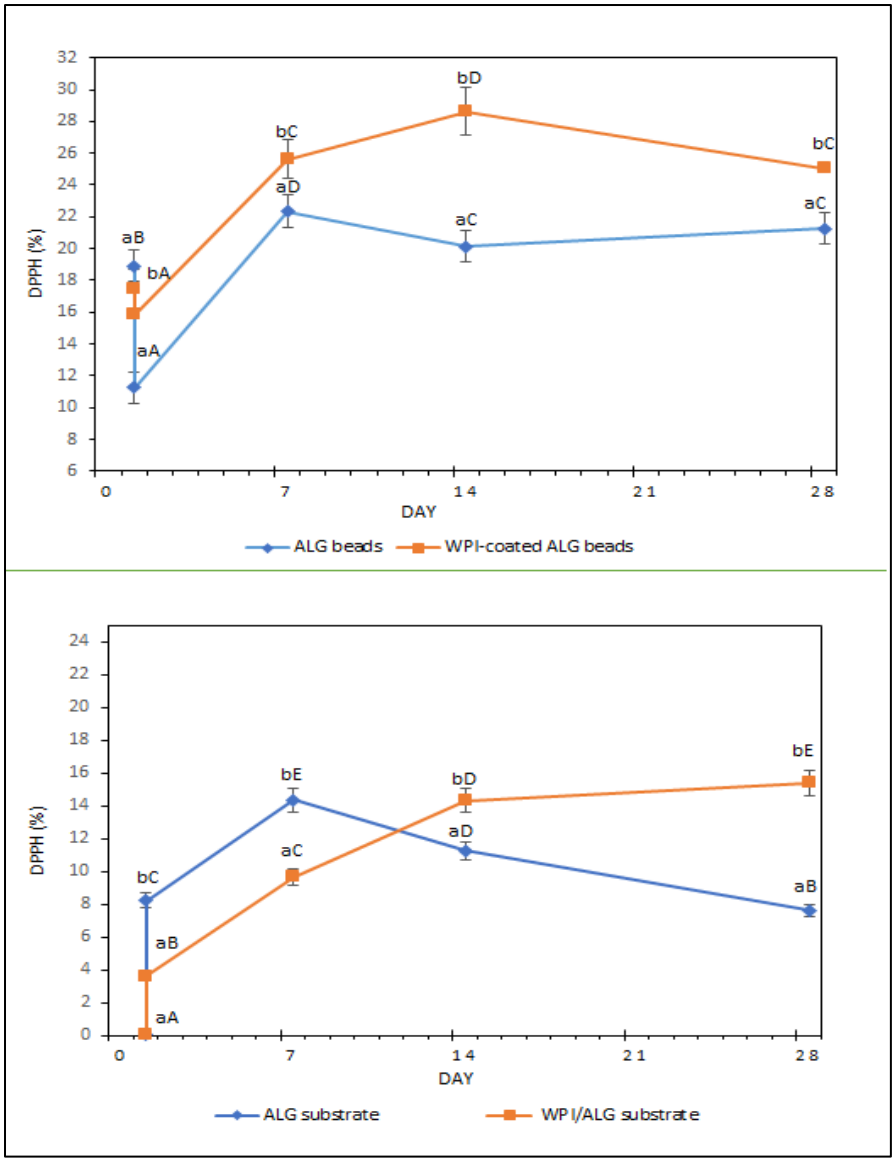

Figure 2. Antioxidant capacity (DPPH) of beads and substrate for sport drink samples with alginate (ALG) and WPI-coated alginate (WPI/ALG) beads, during fermentation and 28 days of storage at 4 ˚C. Means in a column shown with different lowercase letters are significantly different ($p < 0.05$). Means in a raw shown with different uppercase letters are significantly different a row ($p < 0.05$).

In the present study, no significant difference in the pH of the beverage containing uncoated and WPI-coated ALG microcapsules was observed (Table 2). Similar results have been reported by other studies. Peiris et al. (2019) found no difference between the pH of control yogurt and yogurt containing encapsulated Bifidobacterium in an alginate/whey/insulin matrix [19]. In all samples, due to the production of lactic acid by the starter culture, the pH decreased during the storage period. At the end of the storage period, fermented beverages containing coated microcapsules showed a higher pH compared to the samples containing alginate microcapsules. No significant difference in acidity between the samples was found ($p < 0.05$). Despite the higher acidity of the samples containing WPI-coated microcapsules, the pH remained in the range of 4–4.5 due to the buffering properties of whey peptides and amino acids.

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Table 2. pH and titratable acidity values of sport drink samples containing Alginate (ALG) and WPI coated alginate (WPI/ALG) beads during refrigerated storage.

Treatment	Storage	ALG	WPI Coated ALG
pH	1st day	4.81 ± 0.02^{d}	**4.84 ± 0.19^{d}**
	7st day	4.69 ± 0.15^{c}	**4.72 ± 0.12^{c}**
	14st day	4.61 ± 0.06^{bc}	**4.69 ± 0.23^{b}**
	21st day	4.42 ± 0.06^{b}	**4.51 ± 0.06^{b}**
	28st day	4.10 ± 0.12^{a}	**4.21 ± 0.15^{a}**
Acidity (%Acid lactic)	1st day	1.21 ± 1.09^{a}	**1.24 ± 1.09^{a}**
	7st day	1.31 ± 0.09^{ab}	**1.29 ± 0.08^{ab}**
	14st day	1.35 ± 0.03^{ab}	**1.32 ± 0.15^{ab}**
	21st day	1.66 ± 0.03^{b}	**1.70 ± 0.15^{b}**
	28st day	2.54 ± 0.10^{c}	**2.51 ± 0.10^{c}**

Numbers are expressed as mean ± standard deviation. Means in a column shown with different lowercase letters are significantly different ($p < 0.05$).

4-3 - Viability of Encapsulated Probiotics During Storage

The growth of the probiotic culture encapsulated in alginate increased during the first 7 days of storage (Figure 3). Higher growth was observed in microcapsules containing WPH, indicating the effect of bioactive peptides on bacterial growth. The difference in the viability of probiotics in WPI-coated and uncoated microcapsules may be related to the release of bioactive molecules in the uncoated microcapsules, which could lead to less protective effect for the probiotic cells [16]. The lower rate of bioactive peptide release from WPI-coated microcapsules compared to uncoated capsules during storage resulted in an increase in the number of viable cells in the WPI-coated microcapsules. The addition of the WPI coating led to a more stable matrix with less cell reduction, due to the high acidic tolerance and buffering properties of WPI. At the end of the storage period, all samples contained a higher number of probiotics than the recommended level (g/CFU log 6). The highest number of probiotics after 28 days was observed in the samples containing encapsulated and WPI-coated probiotics. Our results are consistent with similar studies. Krasaekoopt et al. (2014) reported that the number of Lactobacillus acidophilus encapsulated in alginate, chitosan, and galactooligosaccharides after 28 days of storage at 4°C was mL/CFU log 1.8 [20]. Brinques et al. (2011) reported a reduction in the number of Lactobacillus plantarum coated with alginate and chitosan in yogurt after 35 days of storage, which was mL/CFU log 0.55 [21].

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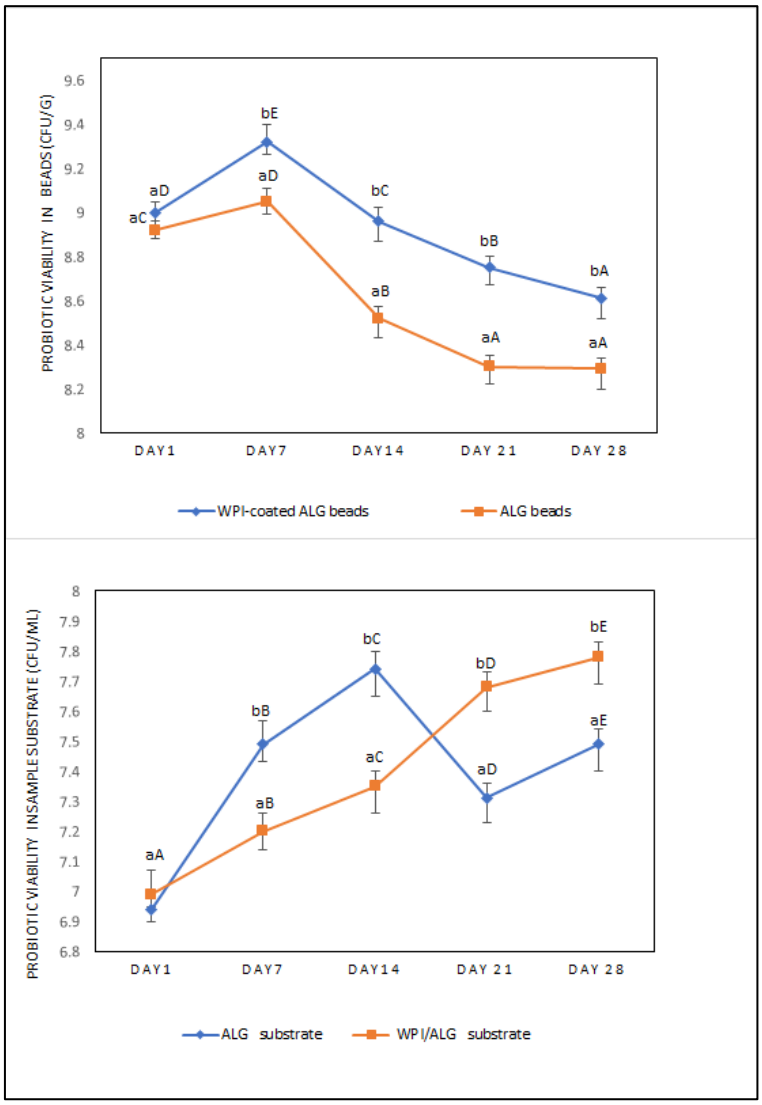

Figure ۳. Viability of probiotic bacteria for encapsulated cells (Log cfu/g) and released cells (Log cfu/ml) in sport drink samples with alginate (ALG) and WPI-coated alginate (WPI/ALG) beads, during 28 days of storage at 4 ◦C. Means in a column shown with different lowercase letters are significantly different ($p < 0.05$). Means in a raw shown with different uppercase letters are significantly different a row ($p < 0.05$).

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5 - Conclusion

In the present study, WPH was used as a natural antioxidant along with probiotic bacteria including Lactobacillus plantarum and Lactobacillus casei encapsulated in an alginate matrix and subsequently coated with WPI in a whey protein-based sports drink. Encapsulation and coating of bioactive peptides and probiotics successfully enhanced the survival of probiotics and reduced the release rate of probiotics and bioactive peptides in the product. After 28 days of storage, the final product contained acceptable levels of bioactive peptides and probiotic bacteria. Considering the global interest in functional foods with added value, this product can be proposed as a probiotic and functional sports drink.

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چکیده

این تحقیق با هدف فرموله‌کردن یک نوشیدنی ورزشی ایزوتونیک فراسودمند برپایه ی پرمیت آب پنیر حاوی پروبیوتیک ها و پپتیدهای آب پنیرانکپسوله شده توسط آلژینات انجام شد. اثر پروتئین آب پنیر هیدرولیزشده به‌عنوان آنتی اکسیدان طبیعی و پروتئین‌های آب پنیر به‌عنوان پوشش میکروکپسول ها در نوشیدنی فرموله شده مورد بررسی قرار گرفت. انکپسول پروبیوتیک ها و پپتیدهای آب پنیر به بهبود خواص مکانیکی کپسول، فعالیت تخمیری و بقای پروبیوتیک‌ها در طول ۲۸ روز نگهداری کمک کردند. کپسول های آلژینات پوشش‌داده‌شده با پروتئین آب پنیر کارایی کپسولاسیون، فاکتور کروی‌بودن و ظرفیت آنتی‌اکسیدانی بهتری را قبل و بعد از تخمیر نسبت به کپسول های بدون پوشش نشان دادند. هیدرولیز پروتئین تأثیر مثبتی بر خواص آنتی‌اکسیدانی و زنده‌مانی باکتری های پروبیوتیک نوشیدنی در طول ۲۸ روز نگهداری داشت. نوشیدنی ورزشی فرموله شده بر پایه آب پنیر می تواند بعنوان یک جایگزین طبیعی و فراسودمند برای نوشیدنی های تجاری موجود باشد.

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