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Innovative Food Science and Bioprocesses

PROBIOTIC ALMOND-BASED BEVERAGE: PROMISING STEP TOWARDS A CIRCULAR BIOECONOMY

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Abstract

The search for waste minimization possibilities and the valorization of by-products are key to good management and improved sustainability in the food industry. Although dairy products still remain at the forefront of probiotic food development, in recent years non-dairy food products have increased in popularity owing to their unique characteristics and advantages. Due to its beneficial properties, caused by the presence of various bioactive compounds, the almond parts that remain after almond milk production can be considered promising sources of ingredients for the development of non-dairy food products.

The aim of this study was to examine the potential and possibility of using a surplus product that remains after almond milk production. The antioxidant properties of the remained surplus product were characterized based on the polyphenol, flavonoid, and anthocyanin content as well as FRAP, DPPH, and ABTS antioxidant activity. The fermented beverage that combines the properties of almonds and probiotic bacteria, formulated using inulin, lyophilized fruit and aroma, was evaluated for physicochemical, microbiological, and sensory properties during cold storage (4 °C, 21 day).

The surplus product that remains after the almond milk production contains 61.4 mg GAE/100g DW, 16.11 mg QE/100g DW, and 0.993 mg CYE/100g DW of polyphenols, flavonoids, and anthocyanins (respectively), and expresses DPPH, FRAP and ABTS antioxidant activity of 71.56 mg DW/mL, 50.47 mg DW/mL and 53.11 mg DW/mL (respectively).

The fermentation of surplus product that remains after almond milk production leads to the production of the beverage with satisfactory values of quality parameters as follows: pH value

of 4,75, titratable acidity 28,8 °SH, syneresis 7,5%, viable cell count of 7.77 log (CFU mL^{-1}), and sensory characteristics value of 10, that is stable during 21 day of cold storage.
A new probiotic almond-based beverage that combines the properties of both almonds and probiotics can be considered as promising step toward the circular bioeconomy.

Keywords: *Almond, beverage, probiotics, circular bioeconomy, antioxidants*

1. Introduction

Almonds (*Prunus dulcis* (Mill.) D.A. Webb) are among the most popular nuts, commonly used as snacks or as ingredients in various types of processed foods, especially in bakery and confectionery products (1). Likewise, plant-based beverages are becoming increasingly popular due to the rise of vegetarianism and consumer awareness of their health effects. Depending on the method of extracting the almond pulp after the production of almond milk, about 15-20% oil, about 35-40% protein, but also numerous other valuable substances remain in it (2). The fact is that today this residue is almost unused, and actually has excellent potential for wide application in the food industry. Although dairy products, like yogurt and cheese as the most commonly used carriers for delivering probiotics to the human digestive tract, remain at the forefront of probiotic food development, non-dairy food products are becoming increasingly popular due to their unique characteristics and advantages, such as meeting the needs of vegetarians, and providing lactose-free or low-cholesterol nutritional value products. In recent years, nuts have been increasingly used to produce new nutritious alternatives to cow's milk. The most common substitutes for dairy products are drinks based on soy, almonds, and rice. In this group of products, the "milk" obtained from almonds is the most interesting for its health effects, including antioxidant effects as well as immunological and cardioprotective properties (3). On the other hand, almond pulp as a by-product that remains after the production of almond milk, represents a reservoir of very valuable substances, due to which it can be considered a surplus product worthy of exploitation. In order to exploit and increase its value, and ensure economic and ecological sustainability, it is necessary to examine the possibility of using almond pulp for the design of innovative added-value products. The aim of this study was to examine the possibility of using almond pulp, as a raw material for the production of a functional fermented almond-based beverage. As part of the study, the antioxidant activity of almond pulp was examined, in order to gain insight into the content and potential of valuable bioactive compounds whose main role is the neutralization of free radicals, and thus the quality

of the starting raw material. After an insight into the quality of the raw material, the possibility of its application in the production of a functional fermented almond-based beverage was examined.

2. Experimental

2.1. Preparation of extracts

Fresh almond pulp sample (1g) was mixed with 70.0 % ethanol (10 mL) and vortexed for approximately 5 min. The extraction was performed at 50°C for 4 h with an orbital shaker under constant rotatory agitation at 200 rpm. The mixture was centrifuged for 5 min at 6000 rpm and the resultant supernatant was separately kept at 4 °C for subsequent assays.

2.2. DPPH radical scavenging activity

Procedure described by Brand-Williams et al. (4) , with slight modification, was employed for DPPH assay. An appropriate amount of extract was mixed with a 0.2 mM solution of DPPH in ethanol, after which the samples were left for 60 min in the dark for the reaction to take place. Absorbance (A) was measured at 517nm. The DPPH antioxidant activity was calculated according to the equation:

Inhibition percentage (%) = (Acontrol – Asample) / Acontrol x 100 (1)

The results are expressed as IC_{50} (mg DW/mL), the concentration of dry matter of the sample necessary to achieve 50% inhibition of DPPH radicals.

2.3. ABTS radical scavenging activity

Procedure described by Re et al. (5) was employed for ABTS assay. Briefly, the ABTS solution was prepared by mixing 7 mM ABTS salt equal proportion with 2.45 mM potassium per sulphate and the same mixture was then put in the dark for minimum 16 h. The solution absorbance was noted at 734 nm and before mixing with extracts, it was adjusted to 0.7. The mixture was put again in the dark for 15 minutes at room temperature. An apropriate amount of extract was mixed with ABTS reagent. The mixture was homogenized and incubated for 15 min in the dark. The absorbance was measured at 734 nm. The ABTS antioxidant activity was calculated according to the equation:

Inhibition percentage (%) = (Acontrol – Asample) / Acontrol x 100 (2)

The results are expressed as IC_{50} (mg DW/mL), the concentration of dry matter of the sample necessary to achieve 50% inhibition of ABTS radicals.

2.4. Ferric ion reducing antioxidant power (FRAP)

Procedure described by Benzie and Strain (6) was employed for FRAP assay. The FRAP working solution was made by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl3·6H2O with ratio 10:1:1. An apropriate amount of extract was mixed with FRAP reagent. The mixture was homogenized and incubated for 30 minutes in the dark. The absorbance was measured at 593 nm. Total reducing capacity of FRAP was determined using the following equation:

Inhibition percentage (%) = (Asample – Acontrol) / Asample x 100 (3)

The results are expressed as IC_{50} (mg DW/mL), the concentration of dry matter of the sample necessary to achieve 50% inhibition of FRAP radicals.

2.5. Determination of total phenolics

Total phenolics content in the sample was determined using Folin-Ciacolteu method as described by Kruawan and Kangsadalampai (7). An appropriate amount of extract was mixed with distilled water, Folin-Ciocalteu reagent and 7.5% Na_2CO_3. The mixture was homogenized and incubated for 120 minutes in the dark. The absorbance was measured at 750 nm. Total phenolics content was calculated based on the gallic acid calibration curve. The results were expressed as mg GAE/100 g DW of the sample based on standard curve equations prepared immediately before sample analysis.

2.6. Determination of total flavonoids

The aluminum chloride colorimetric method was used to determine the total flavonoids content following the procedure employed by Lee et al. (8). An appropriate amount extract was mixed with 96% ethanol, 10% aluminium chloride ($AlCl_3 \cdot 6H_2O$), and sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) (1M). Then the mixture was homogenized and incubated for about 45 min. The absorbance of the solution was measured at a wavelength of 430 nm. Total flavonoids content was calculated based on the quercetin calibration curve. The results were expressed as mg QE/100g DW of the sample based on standard curve equations prepared immediately before sample analysis.

2.7. Determination of total anthocyanin

Total anthocyanin was quantified using a pH differential method with a two-buffer system described by Lako et al. (9). Monomeric anthocyanin pigments change color reversibly with a change in pH. The difference in absorbance of the pigments at 510 nm is proportional to the concentration of the pigment. The appropriate amount of sample was mixed with KCl buffer

pH=1 and $CH_3CO_2Na \cdot 3H_2O$ buffer pH=4.5. The absorbance of the solution was measured at wavelengths of 510 and 700 nm, in relation to the corresponding buffer as a blank, within 50 min of preparation. The results are expressed as cyanidin-3-glucoside equivalent, ie mg CYE/100g DW.

2.8. Preparation of fermentation suspension

The fermentation suspension of almond pulp, with the appropriate content of dry matter (5, 10 and 15%), was pasteurized for 60 minutes at a temperature of 60ºC. After the heat treatment the prepared suspension was cooled to the fermentation temperature (42 °C).

2.9. Fermentation

The fermentation suspension was inoculated with 0.06% (*m/v*) ABT-10 culture (Chr. Hansen A/S, Hørsholm, Denmark), composed of *Streptococcus thermophilus*, *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12, transferred to a water bath where fermentation was performed at a temperature of 42°C until the pH ≈4.5 was reached (150 min), after which the fermentation was stopped by quick cooling of the samples. After cooling, the samples was enriched with lyophilized fruit (strawberry, blackberry, blueberry, raspberry) (Drenovac d.o.o., Arilje, Serbia) in the amount of 5% (*m/v*), 1.7% (*m/v*) of inulin and 0.1% (*v/v*) vanilla flavor. After the initial tests (0 day), the samples were stored for 21 days at a temperature of 4°C, during which they were analyzed for the following parameters in equal time intervals of 7 days.

2.10. Chemical analysis

The titratable acidity was determined by the Soxhlet-Henkel method (10), and the pH value was measured using a pH meter (Inolab, WTW 82362, Wellheim, Germany).

2.11. Microbiological analysis

Microbiological analysis of fermented samples was conducted according to the method described by Bulatović et al. (11), using MRS agar and anaerobic incubation at 37 °C for 48 h for the enumeration of viable cell count of probiotic bacteria.

2.12. Syneresis

Syneresis of fermented samples was determined according to the method described by Bulatović et al. (11). The fermented samples (20.0 mL) were centrifuged at 1000 rpm for 10 min. Collected supernatant was drained, weighed and the following equation was used for syneresis calculation:

$$\text{Syneresis}\,(\%) = \frac{\text{Weight of supernatant (g)}}{\text{Weight of fermented sample (g)}} \times 100\% \qquad (4)$$

2.13. Sensory analysis

Sensory analysis of fermented samples was conducted according to the method described by Bulatović et al. (11), with slight modification, using a 10-point hybrid hedonic scale (12) where 1 - disliked extremely; 5 - neither liked nor disliked and 10 – liked extremely.

2.14. Statistical analysis

The experiments were performed in triplicate. All values are expressed as mean ± standard deviation. Mean values were analyzed using one-way ANOVA (Origin Pro 8 (1991–2007), Origin Lab Co., Northampton, USA). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Antioxidant activity and polyphenol composition of almond pulp

Almond pulp in its composition contains the most of nutrients present in almond seeds and skin. Several investigations on almond seeds and skin extracts revealed the presence of various phenolic compounds, well known to possess excellent antioxidant potential. Antioxidant activity and polyphenol composition of almond pulp are presented in Tables 1 and 2.

Table 1. Antioxidant activity of almond pulp expressed as the minimum inhibitory concentration of antioxidant substances (IC_{50}) necessary to neutralize 50% of free radicals

Sample	Antioxidant activity, IC_{50}		
	DPPH, mg DW/mL	ABTS, mg DW/mL	FRAP, mg DW/mL
Almond pulp	71.56 ± 1.140	53.11 ± 0.245	50.47 ± 0.472

As ABTS and DPPH measure the scavenging capacity of reactive oxygen species, and FRAP determines the metal chelating capacity (14), a combination of assays is recommended for the antioxidant activity analysis. Based on the literature data (14), the almond seeds extract is rich in vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, quercetin, kaempferol, isorhamnetin, delphinidin, and procyanidins B2 and B3. On the other hand, as reported by Monagas et al. (15) almond skin contains a total of 33 compounds corresponding to flavanols, flavonols, dihydroflavonols and flavanones, and other non-flavonoid compounds.

Table 2. Polyphenol composition of almond pulp expressed per 100g of dry weight

Sample	Polyphenol composition in 100g DW		
	Total polyphenols, mg GAE/100g DW	Flavonoids, mg QE/100g DW	Anthocyanins, mg CYE/100g DW
Almond pulp	61.4 ± 1.261	16.11 ± 1.186	0.993 ± 0.045

The results presented in Tables 1 and 2 revealed that there is a strong correlation between total phenolic compounds and the antioxidant capacity of almond pulp. The presented results suggest that total polyphenols are the main compounds responsible for the antioxidant capacity of almond pulp. On the other hand, slightly lower results for the content of flavonoids and anthocyanins are consistent with the literature data (16) that amount of almond skin makes up only about 4% of the almond pulp. Therefore, it can be concluded that most of the antioxidant activity of almond pulp comes from almond seeds, which, as the dominant fraction of almond pulp, represents a reservoir of great potential.

3.2. Optimization of the production process of almond-based beverage

As part of the analysis of the possibility of applying almond pulp in the production of a fermented almond-based beverage, the amount of almond pulp necessary to achieve a maximal viable cell count of probiotic bacteria was optimized. The results are shown in Figure 1.

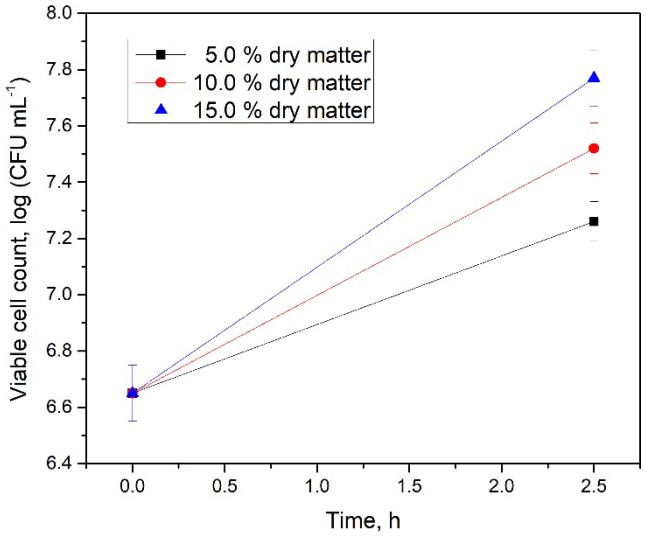

Figure 1. The effect of the amount of almond pulp on the viable cell count of probiotic bacteria in fermented almond-based beverage

As shown in Figure 1, the maximal viable cell count of probiotic bacteria, of 7.7 log (CFU mL^{-1}) was achieved at the almond pulp content of 15% (m/v). The obtained results are in accordance with the fact that by using a larger amount of almond pulp in the fermentation process, a larger amount of carbohydrates dominantly present in the almond pulp, such as

glucose, fructose, sucrose, and raffinose (17), are available to probiotic bacteria. On the other hand, a larger amount of almond pulp implies a larger amount of prebiotics (18) that significantly affect the growth of probiotic bacteria.

3.3. Optimization of the sensory characteristics of almond-based beverage

The changes in acceptability values of fermented almond-based beverages enriched with lyophilized fruits are presented in Figure 2.

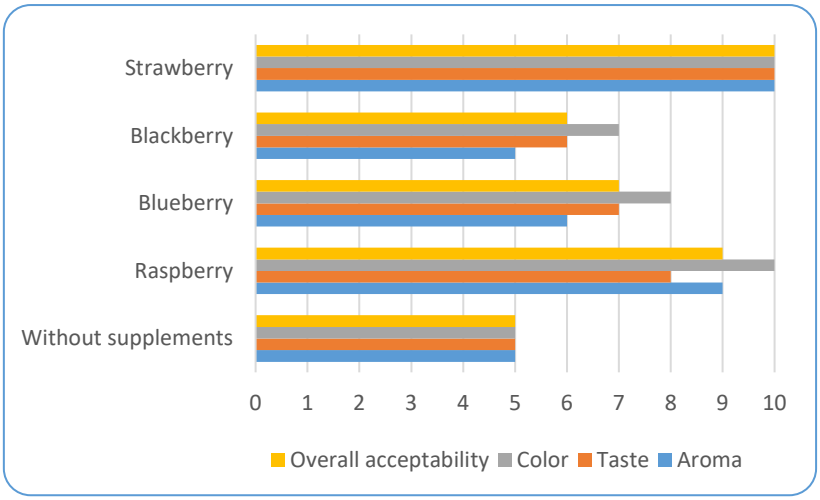

Figure 2. Influence of lyophilized fruits addition on aroma, taste, color and overall acceptability of fermented almond-based beverage

The results presented in Figure 2 indicated that the supplementation by 5% (m/v) of lyophilized fruits significantly ($p < 0.05$) improves the sensory profile of almond-based beverages. The samples enriched with strawberry and raspberry showed high acceptability values of 9 and 10, respectively. The results also show that supplementation with raspberry has a favorable effect exclusively on the color of the drink, while the other ratings are significantly ($p < 0.05$) lower than when using a strawberry. Based on all of the above, it can be concluded that strawberry enrichment is the best way to improve the sensory characteristics of the beverage and thus gain the attention of consumers.

3.4. Stability of the almond-based beverage

Storage stability is the crucial parameter related to the maintaining the quality of product.

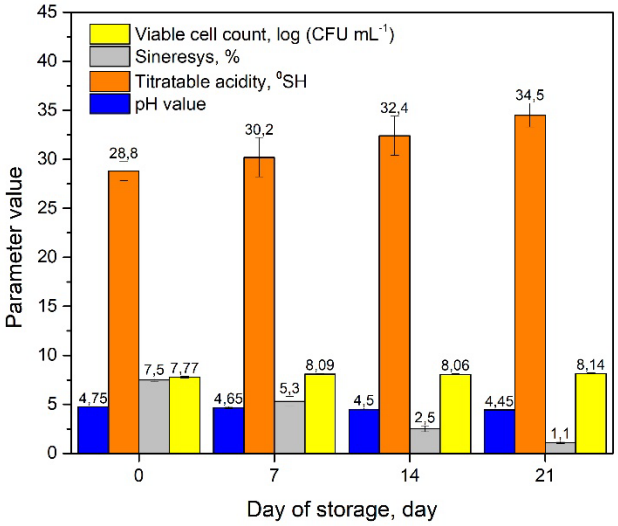

Figure 3. The influence of the storage process on the stability of the almond-based beverage

Based on the results presented in Figure 3, significant ($p < 0.05$) changes in titratable acidity and sineresys were observed between 0 and 21 days of storage. At the end of the storage period, titratable acidity was 34.5 °SH, which is in the range for fermented products (11), while syneresis was at the level of 1.1%, which can be explained by the swelling of the prebiotic fibers present and significant water absorption. On the other hand, the pH value and viable cell count of probiotic bacteria remained stable during the whole storage period which is significantly better than data reported in the literature (19). At the end of the storage period, the fermented almond-based beverage is characterized by a viable cell count of 8.14 log (CFU mL^{-1}) that satisfies the minimum recommended count of viable probiotic bacteria at the time of consumption (20).

4. Conclusion

The main conclusion of the conducted study is that almond pulp, with its excellent content of bioactive substances, represents an excellent substrate for the production of novel almond-based beverage. By fermenting almond pulp, it is possible to produce a sensory acceptable beverage, which contains over 10^8 probiotic bacteria and has a shelf life of at least 21 days.

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