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Anthocyanins decay in pomegranate enriched fermented milks as a function of bacterial strain and processing conditions

M. Cano-Lamadrid [a], L. Trigueros [b], A. Wojdyło [c], Á.A. Carbonell-Barrachina [a], E. Sendra [b, *]

[a] Departamento Tecnología Agroalimentaria, Grupo Calidad y Seguridad Alimentaria, Escuela Politécnica Superior de Orihuela, Universidad Miguel Hernández de Elche, Carretera de Beniel, Km 3.2, Orihuela 03312, Spain
[b] Departamento Tecnología Agroalimentaria, Grupo Industrialización de Productos de Origen Animal, Escuela Politécnica Superior de Orihuela, Universidad Miguel Hernández de Elche, Carretera de Beniel, Km 3.2, Orihuela 03312, Spain
[c] Department of Fruit, Vegetable and Plant Nutraceutical Technology, Wrocław University of Environmental and Life Sciences, 37 Chełmonskiego Street, 51-630 Wroclaw, Poland

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ABSTRACT

Pomegranate juice (PJ) has a potential to be used as a functional ingredient in fermented milks (FM). The aim of this study was to evaluate the quality of FM, stored at 4 °C during 30 days, obtained with 9 starter cultures with the addition of PJ before/after fermentation. The quality parameters studied were microbial counts (MC), anthocyanins (ANCs), color, and pH. All starter cultures maintained counts above 10^6 UFC g^{-1}. Seven ANCs were identified and the predominant one was cyanidin-3,5-di-*O*-glucoside. The ANCs were more stable and decomposed at a minor rate when added before the fermentation step. *Lactobacillus helveticus* led to high MC and provided the highest intensity of the red color due to its low pH. The addition of PJ "before" fermentation was the best choice because it led to the highest and most stable total ANCs content. ANCs experienced significant reductions (mean of 26.1%) during cold storage.

1. Introduction

The popular healthy image of fermented dairy foods has been related to the ingestion of live microorganisms (probiotics) (Kechagia et al., 2013). On the other hand, interest in anthocyanins (ANCs) has enormously increased because of their high antioxidant activity and healthy properties (Wallace & Giusti, 2008; Ścibisz, Ziarno, Mitek, & Zaręba, 2012). Besides, ACNs are the most important group of water-soluble pigments, which color mainly depends on the pH. Fruit juices have a potential to be used as functional ingredients in the dairy sector (Gumienna, Szwengiel, & Górna, 2016; Trigueros, Wojdyło, & Sendra, 2014).

Polyphenols represent the predominant class of phytochemicals of pomegranate juice (PJ), specifically ANCs. In a previous study (Trigueros et al., 2014), it was concluded that adding 40% of PJ to yogurt resulted in a significant enrichment of phenolic compounds, mainly ANCS. The ANCs profile and the content of the main ANC in PJ, cyanidin-3-*O*-glucoside, changed with storage time. Thus, PJ could be considered a good antioxidant ingredient for dairy products, with an attractive and stable color.

Bacterial cultures used in fermented milks (FM) production can carry enzymes that cause hydrolysis of ANCs to less stable aglycones (Buchert et al., 2005) facilitated by the hydrogen peroxide produced by the lactic cultures (Yüksekdağ, Beyath, & Aslım, 2004). The influence of different probiotic cultures on the ANCs profiles and contents in PJ enriched FM is largely unknown. Previously, Ścibisz et al. (2012) determined that the selection of culture for production of other types of FM with ANCs rich fruits is recommended.

Another factor that must be considered is the exact time when the juice should be incorporated in the preparation of the FM, "before" or "after" the fermentation process. In dairy products, caseins tend to associate with the hydroxyl (-OH) group of phenolic compounds (Yuksel, Avci, & Erdem, 2010). Furthermore, this interaction is maximum at the isoelectric point of the protein (O'Connell & Fox, 2001), resulting in a color loss or specific discoloration.

Therefore, the aim of this study was to evaluate (i) the influence

* Corresponding author.
E-mail address: esther.sendra@umh.es (E. Sendra).

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Nomenclature

ANCs	anthocyanins
a^*	redness
b^*	yellowness
FM	fermented milk
HPLC	high performance liquid chromatography
L^*	lightness
LC/MS-QTOF	liquid chromatography-mass spectrometry-quadrupole time-of-flight
MC	microbial counts
PCA	principal component analysis
PJ	pomegranate juice
SMP	skim milk powder
TAC	total anthocyanin content
UPLC–PDA	Ultra-performance liquid chromatography-photodiode array detection

of probiotic starter cultures and (ii) the addition of PJ "before or after" fermentation during 30 days of cold storage at 4 °C on the following parameters: ANCs profile and contents, physicochemical parameters and microbial populations.

2. Material and methods

2.1. Chemicals

Cyanidin 3,5 di-*O*-glucoside and -glucoside, pelargonidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside were purchased from Extrasynthese (Lyon, France) purity was 95–96%. Acetonitrile for ultra-phase liquid chromatography (UPLC; Gradient grade) and ascorbic acid were from Merck (Darmstadt, Germany). UPLC grade water, prepared by HLP SMART 1000s system (Hydrolab, Gdansk, Poland), was additionally filtrated through a 0.22 μm membrane filter immediately before use.

2.2. Plant material

Mature pomegranate fruits, cultivar *Mollar de Elche* were purchased from a local market. The arils were introduced in a blender to obtain freshly prepared PJ; then, the juice was filtered to remove particles.

2.3. Starters

Starter cultures were lactic acid bacteria (LAB) individually used for fermented milk production: (S_1) *Lactobacillus delbrueckii subsp. bulgaricus* CECT 4005, (S_2) *Lactobacillus sakei* subs. *carnosus* CECT 5964, (S_3) *Lactobacillus casei* CECT 475, (S_4) *Lactobacillus curvatus* CECT 5786, (S_5) *Lactobacillus helveticus* CECT 541, (S_6) *Lactococcus lactis subsp. lactis* CECT 4042, (S_7) *Lactobacillus paracasei subs. paracasei* CECT 277, (S_8) *Lactobacillus plantarum* CECT 5785, and (S_9) *Lactobacillus reuteri* CECT 925 (Colección Española de Cultivos Tipo, Universidad de Valencia, Burjasot, España).

2.4. Fermented milk manufacture

Fermented milk was produced following the manufacture method developed by Trigueros et al. (2014), modified by using individual LAB as starter cultures instead of the yogurt cultures used in their research. Fermented milks contained 40% PJ. Two experiments were conducted in this study: (i) adding PJ before fermentation (T_1), and (ii) adding PJ after fermentation (T_2). Fermented milk without PJ addition was used as control for each individual starter culture (T0). Three independent replicates of each experiment were run. Fermented milk were kept in 100 mL Pyrex™ flaks and stored for 30 days under refrigeration. Two flaks were used each sampling day: 1, 10, 20 and 30 days of refrigerated storage.

2.5. Physico-chemical and microbiology analysis

The CIEL*a*b* color space of fermented milk was studied, and the following color coordinates were evaluated: lightness (L*), redness (a*, green-red coordinate), and yellowness (b*, blue-yellow coordinate). Color determinations were made at 12 ± 2 °C by means of a Minolta CM-2002 (Minolta Camera Co., Osaka, Japan) spectrophotometer, with a liquid accessory CR-A70 (Minolta Camera Co., Osaka, Japan), with illuminant D65 and an observer of 10°. The equipment was daily calibrated with the white plate provided by Minolta. pH was determined. MRS agar was used for lactobacilli counts (37 °C, microaerophilia, 48 h) and M17 for lactococci (30 °C, aerobiosis, 48H). Three replicates were run for pH and microbiology, and nine for color.

2.6. Extraction and LC-PDA/MS analysis of anthocyanins

The protocols for identification (LC/MS QTOF) and quantitative (UPLC–PDA) analysis of ANCs were those previously reported by Trigueros et al. (2014). Triplicate analyses of each sample were run. Identification of ANC was carried out using an ACQUITY Ultra Performance LC™ system (UPLC™) with binary solvent manager and PDA detector (Waters Corporation, Milford, MA, USA) and a Micromass Q-TOF Micro mass spectrometer (Waters, Manchester, UK) equipped with electrospray ionization (ESI) source operating in negative and positive mode. Separations of individual phenolic compounds were carried out using a UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters Corporation) at 30 °C. Samples (10 μL) were injected. Analysis was carried out using full-scan, data-dependent MS scanning from *m/z* 100 to 1000. The mass tolerance was 0.001 Da and the resolution was 5000. Leucine enkephalin was used as the internal reference compound during ESI-MS accurate mass experiments and was permanently introduced via the LockSpray channel using a Hamilton pump. The lock mass correction was ±1000 for Mass Window. All TOF-MS chromatograms are displayed as base peak intensity (BPI) chromatograms and scaled to 12,400 counts per second (cps) (=100%). The effluent was led directly to an electrospray source with a source block temperature of 130 °C, desolvation temperature of 350 °C, capillary voltage of 2.5 kV and cone voltage of 30 V. Nitrogen gas used as desolvation gas at a flow rate of 300 L/h. The characterization of the single components was carried out via the retention time and the accurate molecular masses. Each compound was optimized to its estimated molecular mass in the positive mode before and after fragmentation. The data obtained from UPLC/MS were subsequently entered into the MassLynx 4.0 ChromaLynxTM Application Manager software. The runs were monitored at 520 nm for anthocyanins. Retention times and spectra were compared with those of pure standards. Calibration curves at concentrations ranging from 0.05 to 0.5 mg/mL (r2 ≤ 0.9998) were made from cyanidin-3,5-*O*-diglucoside and -glucoside, pelargonidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside as standards. Results were expressed as milligrams per 100 g of sample.