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Full Length Article

Production of fermented skim milk supplemented with different grape pomace extracts: Effect on viability and acidification performance of probiotic cultures

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ABSTRACT

The addition of polyphenolic compounds to probiotic dairy products has been proposed as a promising strategy to enhance the beneficial health effects of milk-derived functional foods. In this study, probiotic fermented skim milk was supplemented with pomace extracts obtained from Pinot Noir, Freisa, Croatina and Barbera grape varieties. Regarding acidification kinetics, the addition of Pinot Noir extract increased the maximum acidification rate (V_{max}) of skim milk by 39.4% compared with control (no pomace extract supplementation). The time required to complete the fermentation ($t_{pH4.5}$ = 3.5 h) was shortened when grape pomace extracts were added to the fermented skim milk. It was also observed that after 28 days of storage at 4 °C, polyphenolic compound supplementations had a positive effect on cell viability of both *Streptococcus thermophilus* and *Lactobacillus acidophilus*. The concentration of polyphenols was also determined in the fermented skim milk samples. These results suggest that *Streptococcus thermophilus* and *Lactobacillus acidophilus* can metabolize the supplemented polyphenols, although not all to the same extent. Moreover, this study demonstrates the feasibility of adding phenolic compounds to probiotic products in order to further improve their functional health properties.

1. Introduction

Lactic acid bacteria (LAB) have traditionally been associated with the fermentation of food and animal feed. LAB are one of the most important microorganisms used in food fermentation, with many LAB strains considered as probiotics. As living microorganisms, probiotics may provide health benefits to the host (when ingested in sufficient amounts) by improving the composition of intestinal microflora [1,2] and by crowding out pathogens that may otherwise cause disease [3].

In the *Streptococcus* genus, there are species recognized as pathogenic and others as probiotics. Whereas pathogenic *Streptococcus* species are associated with human and animal diseases, probiotic ones are important in the dairy industry [4,5]. *Streptococcus thermophilus*, for example, is one of the probiotic bacteria that play an important role in the texture of yogurts and other fermented dairy products [6], especially by the production of exopolysaccharides (EPS) [7]. According to Zhang et al. [8], *S. thermophilus* is responsible for the stabilization effect of EPS on the textural and microstructural properties of fermented skim milk.

Probiotic microorganisms are commonly added into dairy products to provide functional health effects [9]. For instance, the addition of the probiotic *Lactobacillus acidophilus* 593 N to cheese may provide health benefits to consumers through their antagonistic effect against foodborne disease agents, including *Enterococcus faecium* and *Listeria monocytogenes* [10]. In dairy products, the use of co-cultures is very common (e.g., probiotic *Streptococcus* combined with different *Lactobacillus* strains) causing a symbiotic effect. In fact, several authors have observed a more pronounced positive activity of co-cultures in comparison with monocultures in terms of growth, acidification, production of flavor, EPS and proteolysis [11,12].

Grape (*Vitis vinifera*) is one of the world's most important fruit crops, with a global production of around 73 million tons in 2015, of which 274.7 mhl were used to produce wine [13]. This industry generates an enormous amount of biomass, known as pomace, which include grape

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skins and seeds. Grape pomace is known for its notable environmental impact due to its high content in phenols [14,15]. During the last years, the interest in studying polyphenolic compounds has increased due to their antioxidant properties and their likely role in the prevention of several (chronic) diseases. The capacity of antioxidants to protect cells from free radical damages and to prevent diseases, including cardiovascular, cancer and neurodegenerative disorders, have been associated to their anti-inflammatory, anticarcinogenic and antibacterial activities [16–18]. Due to these characteristics, polyphenols have been used in pharmaceutical, cosmetic and food products. According to Moure et al. [19], the antioxidant capacity of phenolic compounds helps to preserve flavor and color, avoid vitamin destruction in foods and protect living cells from oxidative damage.

In fermented dairy products, polyphenols can either be added before the fermentation process as part of the yogurt ingredient mixture or after the fermentation as part of the usual practice of imparting flavor and color agents [20]. Therefore, the combination of polyphenolic compounds with probiotic LAB may represent an innovative biotechnological option to enlarge the market of functional dairy products [21].

It is well known that wines contain a wide range of bioactive compounds including polyphenols, phenolic acids, and flavonoids [22–24]. Of note, grape pomace, a by-product of the wine making process, also contains different polyphenols (e.g., anthocyanins, catechins, glycosides of flavonols and polyphenolic acids) [25].

The aim of this study was to evaluate the potential of four grape pomace extracts as an antioxidant-rich dairy food ingredient. The effects of grape pomace obtained from different wine varieties grown in North and North-West Italian regions (Pinot Noir, Freisa, Croatina and Barbera) on probiotic fermented skim milk production and its composition, including concentrations of inorganic compounds, organic acid, carbohydrates and polyphenol compounds, were evaluated.

2. Material and methods

2.1. Preparation of grape pomace samples

Four grape pomaces from the vinification process of Pinot Noir, Freisa, Croatina and Barbera cultivars were kindly provided by the Province of Alessandria (Piedmont, Italy). The grape pomace samples were obtained after 5–8 days of maceration of the grapes, frozen and stored at −20 °C before analysis. Samples were then dried in an oven (D-82152, MMM Medcenter, Monaco) at 65 °C for 72 h to obtain 4% residual water content [15]. The pomaces were ground using a mixer grinder (MX-AC400, Panasonic, Kadoma, Japan) and the powder samples obtained (0.7 mm) were placed in sealed containers and stored away from light, heat and moisture, to ensure correct preservation of matrix before use, thereby preventing oxidative phenomena that may alter or reduce phenolic content.

2.2. Polyphenol extraction

A laboratory-scale hermetically-closed agitated reactor (Parr 4560, PARR Instrument Company, Moline, USA) that could operate under high pressure and temperature (HPTE) was used to extract polyphenols from the four different grape pomace cultivars. Water was employed as the solvent and the solid to liquid ratio was fixed at 1:10 (w/w). Extraction was carried out at 150 °C for 150 min, in accordance with the methods previously reported by Casazza et al. [26]. In these conditions, the resulting pressure was 9.2 bar. In order to decrease oxidation reactions, air was replaced by N_2 in the reactor chamber at the beginning of the extraction. After the extraction, supernatants were separated from the solid by centrifuging the mixture at 6000 g for 10 min. The total phenolic content of extracts was determined using the Folin-Ciocalteu reagent according to Swain and Hillis [27].

2.3. Skim milk preparation

The skim milk (SM) was prepared in 200 mL-Erlenmeyer flasks by adding 10.4 g of non-fat powder milk (Castroni, Reggio Emilia, Italy) to 69.6 mL of deionized water. SM base was thermally treated at 90 °C for 5 min in a water bath (Grant, Cambridge, United Kingdom), cooled in ice bath and stored at 4 °C for 24 h.

Different volumes of aqueous extracts (3.6 mL of Pinot Noir (SMPN), 3.0 mL of Freisa (SMF), 3.1 mL of Barbera (SMB), and 2.8 mL of Croatina (SMC)) obtained by HPTE were added to the SM to bring the total phenolic concentration to 80 mg/L. This concentration of phenolic compounds was chosen based on the work published by Servili et al. [21]. The yield of total polyphenols was expressed as milligrams of gallic acid per mL of fermented skim milk (mg_{GA}/mL).

2.4. Microbial cultures and growth conditions

Two commercial freeze-dried starter strains (Dupont Danisco, Sassenage, France), *Streptococcus thermophilus* TA040 (St) and *Lactobacillus acidophilus* LAC4 (La), were used in this study. St preculture was prepared by dissolving 3.6 mg of freeze-dried culture in 50 mL of autoclaved (121 °C for 20 min) SM with a total solids content of 10% (w/w). After mixing and activating the pre-culture by incubating at 42 °C for 30 min, 1.0 mL was used to inoculate 80 mL of SM in a 200 mL-Erlenmeyer flask. The La pre-culture was prepared similarly by adding 14 mg of freeze-dried culture to 50 mL of SM. Counts of the two pre-cultures ranged from 6.0 to 6.5 log CFU/mL [12].

2.5. Fermentation process

After inoculation, SM samples were incubated at 42 °C in a controlled water bath until acidity reached pH 4.5. The fermentation kinetics was monitored every 15 min using a pH meter 210 (Hanna Instrument, Padova, Italy). The SM samples were prepared in triplicates and were manually agitated by means of a stainless steel perforated disk-rod that was moved up and down for 60 s. The fermented products were dispensed into 50 mL polypropylene cups, thermally sealed and cooled in an ice bath prior to storage at 4 °C.

2.6. Kinetic parameters

The maximum acidification rate (V_{max}) was calculated as the time variation of pH (dpH/dt), expressed as 10^3 units of pH/min. During the incubation period, the following parameters were calculated: t_{max} (h) as the time where V_{max} was reached and $t_{pH4.5}$ as the time required for the fermented milk to reach pH 4.5.

2.7. Microbial counts

Bacterial strains were counted by pour plate technique under aerobic (for St) and anaerobic (for La) incubation at 37 °C for 48 h as suggested by Oliveira et al. [28]. Working under a laminar flow hood (Faster, Milan, Italy), samples (1.0 mL) were diluted in 9.0 mL of 0.1% sterile Peptone Water (Oxoid, Basingstoke, United Kingdom). The bacterial counts were performed after 1 day (D_1), 7 days (D_7) and 28 days (D_{28}) post–fermentation (storage period). The populations of St in the samples were determined using M17 agar medium (Oxoid, Basingstoke, UK) supplemented with lactose (2.5%, v/v) (Carlo Erba, Val de Reuil, France). La populations were determined using MRS agar (Oxoid, Basingstoke, UK). In order to achieve a modified atmosphere, an anaerobic jar (2.5-l, AnaeroGen™ Anaerobic System, Oxoid, Basingstoke, UK) was used. The jar was fitted with a pressure gauge to control pressure loss.