PharmaNutrition 6 (2018) 64–68

Contents lists available at ScienceDirect

PharmaNutrition

journal homepage: www.elsevier.com/locate/phanu

Full Length Article

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

Pamela Oliveira de Souza de Azevedo[a], Bahar Aliakbarian[b,c], Alessandro Alberto Casazza[c], Jean Guy LeBlanc[d], Patrizia Perego[c], Ricardo Pinheiro de Souza Oliveira[a,*]

[a] Department of Biochemical and Pharmaceutical Technology, University of São Paulo, São Paulo 05508-900, Brazil
[b] Department of Supply Chain Management, Michigan State University, Midland 48640, USA
[c] Department of Civil, Chemical and Environmental Engineering, University of Genoa, Genoa I-16145, Italy
[d] CERELA-CONICET, San Miguel de Tucuman, C.P. 4000, Argentina,

ARTICLE INFO

Keywords:
Probiotic
Skim milk
Pomace extracts
Lactobacillus acidophilus
Polyphenolic compounds

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

* Corresponding author.
E-mail address: rpsolive@usp.br (R.P. de Souza Oliveira).

https://doi.org/10.1016/j.phanu.2018.03.001
Received 18 December 2017; Received in revised form 2 March 2018; Accepted 3 March 2018
Available online 05 March 2018

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.

Table 1
Acidification kinetics of skim milk fermented by co-culture of *L. acidophilus* (La) with *S. thermophilus* (St).

Runs	V_{max} (10^{-3} pH units/min)	t_{max} (h)	t_f (h)
Pinot Noir	17.7 ± 0.35^{d}	2.25 ± 0.04^{a}	3.50 ± 0.07^{a}
Freisa	14.8 ± 0.30^{b}	2.75 ± 0.06^{b}	5.00 ± 0.10^{c}
Croatina	16.7 ± 0.33^{c}	3.00 ± 0.06^{c}	4.00 ± 0.08^{b}
Barbera	13.5 ± 0.27^{a}	3.25 ± 0.06^{d}	5.50 ± 0.11^{d}
Control	12.7 ± 0.25^{a}	5.50 ± 0.11^{e}	10.30 ± 0.21^{e}

Vmax, maximum rate of acidification; tmax, time to reach Vmax; tf, time to complete the fermentation (pH 4.5). Different letters in the same column mean statistically significant difference among the values of the same parameter, according to the test of Tukey ($P < 0.05$). indicates standard deviations with respect to the mean values of quadruplicate runs.

2.8. Statistical analysis

The experimental data are presented as mean values ± standard deviations. Mean values of concentrations were analyzed by one-way analysis of variance (ANOVA) using Statistica Software 12 (Tulsa, Oklahoma, USA). Results were compared using the Tukey's post-hoc test and considered significantly different at $p < 0.05$.

3. Results and discussion

3.1. Acidification kinetics

St and La (starter cultures) were used to ferment SM supplemented with extracts from pomace obtained from four different grape cultivars (Pinot Noir, Freisa, Croatina, and Barbera). The pomace extracts contained different types and amounts of polyphenols.

After inoculation of SM with starter cultures, the acidification rates (V_{max}) ranged from 12.7×10^{-3} pH units/min (SMC) to 17.7×10^{-3} pH units/min (SMPN) (Table 1). The addition of a polyphenol-rich pomace extract increased the acidification rates; for instance, supplementation of SM with Pinot Noir pomace extract showed the greatest effect, raising V_{max} by 39.4% compared with control (i.e. SM without any grape pomace extract).

Furthermore, in SMPN, V_{max} was 19.6, 6.0 and 31.1% higher compared with SMF, SMC and SMB, respectively. SMPN also had the shortest time to reach the maximum acidification ($t_{max} = 2.25$ h).

Fig. 1 shows the acidification curves of binary co-cultures of St and La (St-La) in SM supplemented with one of the four different grape pomace extracts. Here, SMPN had a significantly shorter fermentation time ($t_{pH4.5} = 3.5$ h), i.e., 43.0, 14.3, 57.1 and 194.2% faster than SMF, SMC, SMB and SM, respectively. These results suggest that the acidification profile of SM depends not only on the interactions between St and La, but probably also on the supplementation of polyphenols (e.g. in the form of grape pomace extract). Even though the initial concentration of polyphenols were the same in all SM preparations regardless of pomace source (80 mg/L), the relative amount of polyphenol types, i.e., flavonoids, non-flavonoids and anthocyanins, may differ between the four grape-derived pomace supplements. This is in accordance with previous observations by Servili et al. [21] who reported that functional milk beverage fortified with olive vegetation water phenolic extract (OVWPE) had the same phenolic composition at the end of fermentation, but the relative amounts of different polyphenols varied. The authors also noted that the addition of OVWPE had to be compatible with the microorganisms during milk fermentation, showing the importance of interaction between starter cultures and phenolic compounds.

Although carbohydrate levels of pomace extracts were not quantified, one might speculate that the addition of polyphenol compounds affected the acidification kinetics, and consequently the fermentation time, of SM samples possibly due to the amount of sugars present in the polyphenolic extracts used for SM supplementation. When a polyphenolic extract contains a lower concentration of sugars, the carbon source available for bacterial growth is also lower, thus resulting in shorter final fermentation time. Similar results were reported by Servili et al. [21] using OVWPE. In their study, the authors observed that the addition of 200 mg/L OVWPE to functional milk beverage resulted in 10 h fermentation time; in contrast, when the concentration of OVWPE was reduced to 100 mg/L, the fermentation ended after only 7 h.

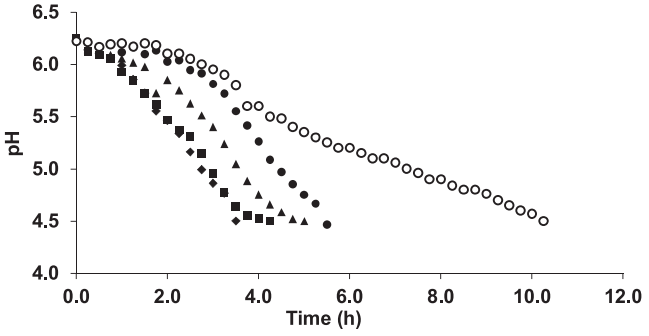

Fig. 1. Acidification curves of skim milk supplemented with different grape pomace extracts and fermented at 42 °C by binary co-cultures of *S. thermophilus* and *L. acidophilus* until reaching pH 4.5. Legends: ○ Control; ● Barbera; ▲ Freisa; ■ Croatina; ♦ Pinot Noir.

3.2. Microbial counts

At the end of fermentation time (D_{28}), St count in the control (SM without phenolic supplementation) was 7.9 log CFU/mL (Fig. 2), equivalent to a reduction of 9.2% from the beginning of the fermentation (D_1). St had the highest concentration (8.9 log CFU/mL) at the end of fermentation in SMPN (Fig. 2) and, on average, there was a decrease of 5.6% of St growth in SMPN, SMF and SMC after 28 days of storage at 4 °C. On the other hand, St viability with SMB decreased by approximately 7.0%. These results suggest that the addition of grape pomace extract supported the viability of St during storage. Dos Santos et al. [29] also observed the same protective effect of grape pomace extract-derived additives on the storage of probiotic cultures.

Pinot Noir extract led to the highest La count (Fig. 2) during the storage period (9.3 log CFU/mL in D_1, 9.0 log CFU/mL in D_{14} and 8.9 log CFU/mL in D_{28}). In SM, after 28 days of storage, La decreased by 10.3% (reaching 6.8 log CFU/mL). According to Kurmann and Rasic [30], probiotics in the food products should be at least 6.0 log CFU/mL to confer any health beneficial effects to the host. In all the samples, including the control sample, the St and La counts were higher than the recommended dose. Moreover, the reductions in La in SMPN, SMF, SMB and SMC at D_{28} were approximately 4.1, 7.8, 9.1 and 7.6%,

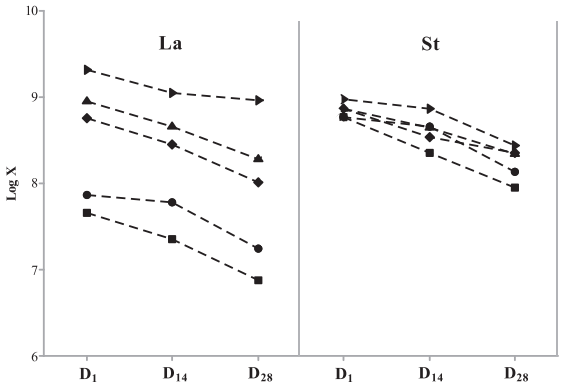

Fig. 2. Counts of binary co-cultures of *S. thermophilus* and *L. acidophilus* in fermented skim milk supplemented with different grape pomace extracts during 28 days of storage at 4 °C. Legends: X (CFU/mL); ■ Control; ● Barbera; ♦ Croatina; ▲ Freisa and ▶ Pinot Noir.

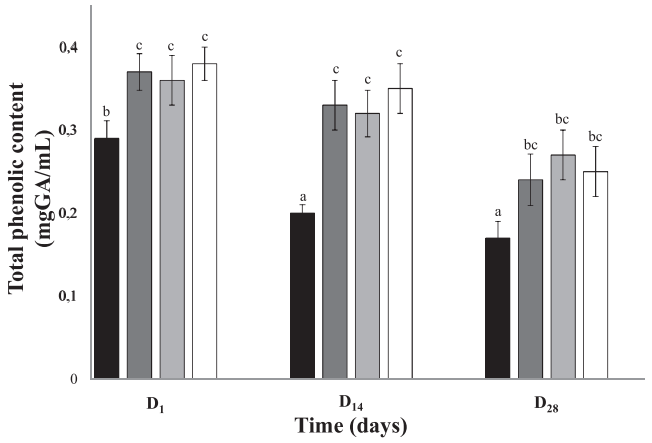

Fig. 3. Concentration of polyphenolic compounds in fermented skim milk supplemented with different grape pomace extracts. Legends: ■ Pinot Noir; ■ Freisa; ■ Croatina; □ Barbera. Different letters in the same bar indicates statistically significant differences ($p < 0.05$).

respectively, and hence much lower than the reduction observed in the control group. These results further indicate that the grape extracts may provide a protective effect on the viability of both probiotic strains (La and St) during storage.

Sun-Waterhouse et al. [31] added apple polyphenols to yogurts and found that these supplements promoted the growth of both *S. thermophilus* and *L. bulgaricus*. In their subsequent work, the authors further showed that the addition of blackcurrant polyphenol extract to yogurts increased the *Streptococcus* count by 40–50 times compared to *L. bulgaricus* [32]. On the other hand, Chouchouli et al. [33] reported that the supplementation of full-fat and non-fat yogurts with two kinds of grape seed extracts (Moschofilero and Agiorgitiko varieties) did not cause significant change in the populations of LAB (*S. thermophilus* and *L. bulgaricus*) compared with non-supplemented yogurts. Tabasco et al. [34] showed that microbial growth depends on both the polyphenol extract composition and the bacterial strain; *S. thermophilus*, *L. fermentum*, *L. acidophilus* and *L. vaginalis* strains appeared to have remarkable sensitivity to phenolic extracts, including the monomeric-rich grape seed extracts at a concentration of 0.25 mg/mL, whereas *L. plantarum*, *L. casei*, and *L. bulgaricus* reached maximal growth.

The beneficial effect of phenolic compound supplementation on LAB grown in milk was also reported by Servili et al. [21]. The growth of LAB increased by 2.5 log CFU/mL, demonstrating the capacity of LAB to metabolize polyphenols [35].

3.3. Polyphenolic compounds in fermented milk

With respect to polyphenolic compound concentrations in fermented milk, values ranged from 0.17 mg_{GA}/mL (SMPN – D_{28}) to 0.38 mg_{GAE}/mL (SMB – D_1) (Fig. 3). Similar results (0.17, 0.20 and 0.29 mg_{GA}/mL) have been reported by Tabasco et al. [34] in skim milk fermented by St-La. Results from the same authors further suggest that some LAB are able to metabolize (−)-epicatechin, (+)-catechin and flavan-3-ol gallates. The concentrations of such compounds decreased after incubation with *L. plantarum* IFPL935. Further, among 19 LAB and 3 bifidobacteria strains which were suggested to this study, *L. plantarum* IFPL935, *L. casei* IFPL7190, *B. breve* 26M2 and *B. bifidum* HDD541 reached maximum concentrations in the presence of grape seed extracts.

The reduction of the polyphenol concentrations in SM and the improvement of LAB growth can probably be explained by the presence of enzymes responsible for metabolic turnover of phenolic compounds. Tabasco et al. [34], for example, reported that the presence of galloyl-esterase, decarboxylase and benzyl alcohol dehydrogenase activities of *L. plantarum* IFPL935 led to the hydrolysis of grape seed polyphenols, subsequently leading to the formation of gallic acid, pyrogallol and catechol, respectively.

Many polyphenols in food are hydrolyzed by the colonic microbiota and/or by intestinal enzymes prior to absorption, suggesting that using phenolic compounds as a food supplement may indeed be a viable strategy for improving the health benefits of fermented milk products used in human and animal nutrition [36].

This current study showed the positive effect of grape pomace extracts on the growth of probiotic strains, and its potential use as an additive in fermented milk products. However, the phenolic composition of pomace extracts is an important consideration given the varying enzymatic profiles among LAB.

4. Conclusions

The results of this study confirm that grape pomace extracts which contain phenolic compounds can be successfully incorporated into skim milk to improve LAB growth during the fermentation process. Of note, phenolic compounds of all four tested grape pomace extracts (Pinot Noir, Freisa, Croatina and Barbera) were metabolized by LAB. The highest breakdown rate of phenolic compounds was observed with Pinot Noir pomace extract. The concentrations of *Lactobacillus acidophilus* LAC4 (La) and *Streptococcus thermophilus* TA040 (St) were lower by 10.3% and 9.2%, respectively after 28 days of storage post-fermentation without grape pomace supplementation. In the presence of Pinot Noir extract no growth reduction for La and only a decrease of 5.6% for St were observed, showing a possible protective effect of this phenolic extract on LAB survival. Adding grape pomace extracts significantly reduced fermentation time. Results of this study indicate an opportunity for development and/or improvement of processing and production of functional dairy food products using grape pomace extracts. Future studies should focus on illuminating the interaction between the phenolic composition of grape pomace extracts and the enzymatic profiles of different LAB.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

The authors are grateful to the financial support provided by CAPES (Coodination for the improvement of higher education personnel) for PhD fellowship of P.O.S. Azevedo (Process no 1560096) and CNPq (National Council for Scientific and Technological Development), Brazil. The editorial assistance of ACADELION Scientific and Medical Communications is acknowledged.

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