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Addition of Strawberry, Raspberry and "Pitanga" Pulps Improves the Physical Properties of Symbiotic Yoghurts

Luciana Bueno, Thamires M. S. Silva, Natália P. Perina, Cristina Bogsan, Maricê N. Oliveira*

Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Av. Prof Lineu Prestes, 580, Bl.16, 05508-900, São Paulo, Brazil

monolive@usp.br

The health benefits offered by probiotic fermented milks can be increased by adding native fruit pulps. Red fruits contain ellagic acid (EA) and ellagitannins, interesting substances due to their powerful antioxidant properties and other beneficial biological activities, such as cardio protective effects, selective growth inhibition of human pathogenic bacteria, α-amylase inhibition of angiotensin I converting enzyme, and proliferative activities against several different cancer cells. This study aimed to improve the physical properties of symbiotic yoghurts through their supplementation with a mixture of red berry pulps: strawberry, raspberry and "pitanga". The *simplex-centroid* design was used for mixture modelling. The design included seven batches/trials: three supplemented with each type of fruit pulp, three corresponding to the binary mixtures and one supplemented with the combination of the three pulps. A control experiment was prepared without addition of fruit pulp. Processed milk bases were fermented at 42 °C until pH 4.7 by using a starter culture blend that consisted of *Streptococcus thermophilus* (TA040), *Lactobacillus bulgaricus* (LB340) and *Lactobacillus acidophilus* (LA140). Subsequently, milk base (80 %) was blended with a pulp preparation (20 %) and other ingredients according to the experimental design. Physico-chemical analyses, enumeration of viable bacteria, colour and rheological characteristics of the yoghurts were evaluated 36 h after preparation and 21 days after cold storage. Three regression models were adjusted to the results (linear, quadratic and cubic special). The results showed that the addition of a mixture of red fruit pulps affected pH and counts of probiotic bacteria, which slightly decrease during storage. Addition of the three red berry pulps simultaneously enhanced the colour and rheological properties of probiotic yoghurts when compared to the control. It was possible to estimate the optimum mixture compositions of the three fruit pulps that increase EA contents in probiotic yoghurts.

1. Introduction

Probiotics are living microorganisms that, confer numerous health benefits to the host when they are supplied in adequate amounts (FAO/WHO, 2002). These microorganisms interact with intestine bacterial flora favoring the presence of beneficial bacteria and reducing the concentration of undesirable bacteria and microorganisms (Fuller, 1991), therefore improving intestinal balance. Prebiotics are substances that, given their structure, they are only fermented in the colon by endogenous bacteria that improve bowel function by stimulating the growth of beneficial bacteria. The presence of beneficial bacteria has specific effects on gastrointestinal physiology, mineral bioavailability, immune system, genesis regulation of tumors and serum cholesterol. From all available prebiotics, only fructooligosaccharides (FOS) and inulin can be considered as active components of functional foods (Douglas and Sanders, 2008; Roberfroid et al., 2010; Oliveira et al., 2012). Consequently, those products containing probiotics and prebiotics in their formulation (symbiotic products) are increasing in popularity and acceptance worldwide (da Cruz et al., 2007; Siro et al., 2008; Oliveira et al., 2011; Oliveira, Perego, et al., 2011).

Numerous studies have shown that several fruits increase the nutritional value to food, because they are potential beneficial for human health (Espírito Santo et al., 2011). Among the bioactive compounds present

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in fruits, the elagitanimos and the ellagic acid (EA), have shown to have physiological and metabolic functions. Scientific interest in EA and ellagitannins is growing , due to their powerful antioxidant properties and other biological activities, such as cardioprotective effects (*in vitro* and *in vivo*), selective inhibition of growth of human pathogenic bacteria, inhibition of α-amylase converting enzyme angiotensin I (ACE) and antiproliferative activities against several different cancer cells effects (Hassimotto et al., 2008; Hassimotto and Lajolo, 2011).

Based on these considerations, the present study aimed to improve the physical properties of symbiotic yoghurts throught their supplementation with a mixture of red berry pulps, strawberry, raspberry and "pitanga", and using an experimental design of *simplex-centroid* for mixture modelling.

2. Materials and methods

2.1 Experimental design and statistical analyses

Processed milk bases (10 % w/v) were fermented at 42 °C until pH 4.7 using *Streptococcus thermophilus* (TA040, DuPont Danisco, Dangé-Saint-Romain, France), *Lactobacillus bulgaricus* (LB340, DuPont Danisco, Dangé-Saint-Romain, France) and *Lactobacillus acidophilus* (LA140, DSM, Moorebank, NSW, Australia). The acidification kinetics were followed by CINAC - *Cinétique d' acidification* (Ysebaert, Frépillon, France) during the fermentation. Subsequently, milk base (80 %) was blended with a pulp preparation (20 %), containing inulin, sucrose, sucralose, acesulfame-K, maltodextrin in a Thermomix (Vorwek & Co. KG, TM31, Wuppertal, Germany).

A *simplex-centroid* design for mixture modelling was applied (Table 1). The method included seven batches/trials: three supplemented with each type of fruit pulp, three corresponding to the binary mixtures and one supplemented with the combination of the three pulps. A control experiment was prepared without fruit pulp.The results of EA after 36 h and 21 days of cold storage at 4 °C were evaluated. Multifactorial analysis of variance and mean comparison tests were applied using Statistica 11.0 (Statsoft, Tulsa, USA). Means were compared using the Newman Keuls test using $p \leq 0.05$. Residual analysis, the coefficient of determination (adjusted R^2), the significance of the models and the lack of fit were used to check the quality of the models.

2.2 Physico-chemical analyses

The content of protein, total solids and fat , as well as the density of the milk bases were determined using an ultrasonic milk analyzer Ekomilk (Eon Trading, Bullgary), and replicated 10 times (Venturoso et al., 2007). The pH was determined in duplicate after 36 h and 21 days of storage at 4 °C, using a pH meter model Q-400M1 (Quimis, Brazil).

2.3 Enumeration of viable bacteria

Enumeration of the yoghurt and probiotic microorganisms was made after 36 h and on day 21 after fermentation in four replicates according to Antunes et al. (2007) and Saccaro et al. (2011).

2.4 Colour and rheological parameters

The colour analysis was performed throughout a colorimeter Hunter Lab, model Colour Quest XE (Reston, Virginia, United States). Lightness (L*), redness (a*) and yellowness (b*) were obtained using CIELAB scale and chroma (c*) was calculated by $c^* = (a^2 + b^2)^{1/2}$ (Rosso and Mercadante, 2007).

The rheological parameters were determined for 5.0 ml of sample, at 5 ºC using a cone and a plate rheometer (RVDV III model; Brookfield Engineering Laboratories, Stoughton, USA). The speed was set from 10 to 250 rpm, with increases of 10 rpm every 30 seconds, with a cylinder CP 40. The description of the rheological behavior was performed using the rheological model of Ostwald-de Waele ($\tau = k\ y^n$) in Excell software (Basak and Ramaswamy, 1994; Benezech and Maingonnat, 1994). Results were obtained in triplicate and expressed as Pa^{sn}.

2.5 Determination of ellagic acid

Ellagic acid was determined by HPLC using autoinjector SIL 20AC (Shimadzu, Tokyo, Japan) and a cation exchange column (Phenomenex C-18, G 250x4.6 mm – 5 µm) at 40 °C. The mobile phase consisted of solvent A (H_2O-0. 1 % formic acid) and solvent B (ACN 0.1 % formic acid) and the flow rate was 1.0 ml / min (pump LC-20AD). Each measurement was expressed as the average of two replicas. The identification was made on the basis of the spectra and the retention time, and the quantification was made using external standard calibration curves of area versus concentration. The calibration curve was established from the standard EA at a concentration between 0.5-10 µg.mL^{-1}. The equation $Y = 144862X^2 - 402656X + 330065$ ($r2 = 0.9944$) corresponds to EA. The analyses were performed at the Analytical Center of the Institute of Chemistry of the São Paulo University, São Paulo, Brazil.

3. Results and discussion

3.1 Physico-chemical characteristics, fermentation profile and counts of viable cells

Milk bases employed in this study were skimmed, and had 11.1 ± 0.08 g.100 g^{-1} of total solids, 4.00 ± 0.02 g.100 g^{-1} of protein, and 1.034 ± 0.29 g.mL^{-1} of density, values according to BRASIL (2011). Time to reach pH 4.7 was 5.19 ± 0.12 h with a maximum acidification rate of 19.55 ± 0.79 upH.min^{-1}. Fermentation time using *L. acidophilus* (LA 140) plus yoghurt cultures was higher than that observed by Oliveira, Florence, et al. (2011) in co-cultures of *L. acidophilus* LAC4 and *S. thermophiles (*TA040), whereas acidification rates were similar.

Initial average pH of milk bases was 6.45 ± 0.29. After fermentation, pH reached 4.30 ± 0.04, and it was significantly different than control and symbiotic fruity yoghurts ($p \leq 0.05$). The evolution of the pH value of symbiotic yoghurts after 36 h (d2) and 21 days (d21) of storage showed a decrease in pH between the end of fermentation (an average of 0.40 pH units), and it remained stable at 4.30 ± 0.06 (fruity yoghurts), and 4.42 ± 0.01 (control) after 36 h of storage (Table 1). The post-acidification was more pronounced at d2 for yoghurts containing equal amounts of berry pulps. The values of pH during storage were not so far from those reported by Moreira et al. (2000), who analyzed samples of milk fermented by different strains of yoghurt cultures or in probiotic yoghurts enriched with passion fruit fiber (Espirito-Santo et al., 2013).

After preparation of red berry yoghurts, average initial counts of *S. thermophilus*, *L. bulgaricus* and *L. acidophilus* were 9.21 ± 0.25, 7.68 ± 0.63 and 7.31 ± 0.77 log cfu.mL^{-1}, respectively. The mixture of fermented milk base with fruit pulps slightly affected the bacteria counts. *S. thermophilus* was found in higher numbers than other bacteria remaining stable after 21 days of cold storage. However, the average counts of *L. bulgaricus* and *L. acidophilus* decreased to 0.20 and 0.74 log cfu units, respectively, during storage. At the end of shelf-life, counts reached 9.22 ± 0.09, 7.49 ± 1.10 and 6.58 ± 0.42 log cfu.mL^{-1} respectively for *S. thermophilus*, *L. bulgaricus* and *L. acidophilus*. Counts of *L. acidophilus* were enhanced at d21 for yoghurts having the same proportion of strawberry and “pitanga” pulps (Table 1). Although *L. acidophilus* counts were low, these results agree with those of Damin et al. (2008), who detected that *L. acidophilus* enumerations were below the legislation limit in St–La fermented milks. Nevertheless, *S. thermophilus* enumerations were higher than 7.0 log cfu.mL^{-1} in the products after 21 days of cold storage, which made them suitable for commercialization.

3.2 Colour and rheological characterization

The colour and rheological parameters of fruity yoghurts are illustrated in Table 1. It can be noticed that at d2, the luminosity (L*) ranged from 67.32 to 68.28, being lower than the control yoghurt. In contrast, b* and c* values, that varied from 15.73 to 31.61 and from 16.87 to 31.98, respectively, were higher than the control. These colour attributes were stable after storage. Besides, a* varied significantly after 36h of preparation as well as 21 days after cold storage ($p \leq 0.05$). Yoghurts with raspberry pulps showed the highest value of redness (6.05), whereas those with equal proportions of raspberry and “pitanga” had the lowest value of redness (3.04). After the cold storage, the intensity of redness decreased almost 62 %.

At d2, values of consistency index ranged from 0.60 to 3.93 Pa.s^n while the control had 1.89 Pa.s^n. After 21 days of cold storage, consistency indexes increased for all yoghurts, except for the control and the yoghurts prepared with strawberry and raspberry pulps. Determination coefficients of rheological parameters were greater than 0.70 showing an adequate fit of the flow curves by Ostwald – de Waele model. All yoghurt samples investigated showed normal rheological characteristics of non-Newtonian behavior, characterized by varying flow behavior indexes from 0.49 to 0.90 at d2 and 0.62 to 0.90 at d21, all values lower than one. These results were consistent with those described by Ramaswamy and Basak (1991), Damin et al. (2008), Penna et al. (2001), and Penna et al. (2006).

3.3 Ellagic acid profile and optimization

The total EA content in fruits pulps were respectively 4.205 ± 0.051 μg.mL^{-1}, 6.492 ± 0.000 μg.mL^{-1} and 4.205 ± 0.022 μg.mL^{-1} respectively for strawberry, raspberry and "pitanga". In spite of the fluctuation, these content values allowed us to classify the raspberry as the richest source of EA among the studied fruits. In contrast, the total EA content of fruity yoghurts was significantly different and varied from 1.112 to 5.008 μg.mL^{-1}n in d2, and from 1.533 to 6.679 μg.mL^{-1} in d21 ($p \leq 0.05$).

It was possible to estimate the optimal EA contents in probiotic yoghurts supplemented with red berry pulp by response surface methodology (Table 2). The values and signals of the linear coefficients obtained for each response showed that all components contributed to increase the EA contents (positive β_1, β_2 and β_3 at d2 and d21) (Table 2). However, different effects were observed when combining strawberry and "pitanga" pulps. Significant and negative interactions of the three pulps were observed (β_{123}).

Table 1: Newman-Keuls means[1] for pH, counts of viable bacteria (log cfu.mL^{-1}), colour and rheological attributes in symbiotic yoghurts and control after storage for 36 h (d2) and 21 days (d21) at 4°C.

Time of cold storage	Trial	Proportion of berry pulps	pH	Counts of viable bacteria			Colour attributes				Rheological attributes	
		(x_1, x_2, x_3)		St	Lb	La	L*	a*	b*	c*	K (Pa.sn)	N
d2	1	(1, 0, 0)	4.31^{b}	9.68^{b}	6.41^{ab}	7.06^{b}	68.28^{c}	1.38^{b}	17.41^{a}	17.46^{a}	1.35^{e}	0.84^{c}
	2	(0, 1, 0)	4.30^{b}	9.40^{ab}	8.35^{b}	7.21^{b}	66.33^{a}	6.05^{e}	15.73^{a}	16.85^{a}	0.60^{b}	0.90^{d}
	3	(0, 0, 1)	4.23^{b}	9.16^{a}	7.42^{ab}	9.04^{c}	67.64^{b}	4.86^{h}	31.61^{c}	31.98^{c}	0.80^{c}	0.80^{c}
	4	(½,½, 0)	4.33^{a}	9.04^{a}	7.77^{b}	6.97^{b}	67.99^{c}	3.39^{e}	16.76^{a}	17.09^{a}	3.93^{l}	0.49^{a}
	5	(½, 0, ½)	4.33^{b}	9.11^{a}	7.96^{ab}	6.96^{b}	67.06^{ab}	5.54^{j}	23.59^{b}	24.23^{b}	1.12^{d}	0.79^{c}
	6	(0, ½, ½)	4.29^{b}	8.96^{a}	7.83^{ab}	6.98^{b}	67.70^{b}	3.04^{d}	24.84^{b}	25.03^{b}	1.40^{e}	0.81^{c}
	7	(⅓,⅓,⅓)	4.22^{a}	9.12^{a}	8.03^{b}	6.97^{b}	66.25^{a}	5.20^{f}	22.51^{b}	23.10^{b}	1.40^{e}	0.81^{c}
	Control	-	4.45^{c}	9.41^{ab}	7.81^{b}	7.06^{b}	82.04^{d}	6.55^{m}	22.92^{b}	23.84^{b}	1.89^{g}	0.77^{c}
d21	1	(1, 0, 0)	4.37^{b}	9.34^{a}	7.86^{ab}	6.38^{a}	68.26^{c}	0.01^{a}	18.43^{b}	18.43^{a}	1.74^{f}	0.88^{d}
	2	(0, 1, 0)	4.28^{b}	9.34^{a}	8.65^{b}	6.88^{b}	66.79^{a}	3.61^{f}	17.20^{a}	17.57^{a}	3.09^{j}	0.62^{b}
	3	(0, 0, 1)	4.25^{ab}	9.27^{a}	9.02^{c}	6.69^{a}	67.06^{ab}	3.82^{f}	29.77^{b}	30.01^{c}	2.18^{h}	0.78^{c}
	4	(½,½, 0)	4.32^{ab}	9.12^{a}	6.45^{ab}	6.36^{a}	68.31^{c}	1.22^{b}	17.99^{a}	18.03^{a}	1.08^{d}	0.90^{d}
	5	(½, 0, ½)	4.34^{b}	9.14^{a}	7.34^{ab}	7.34^{b}	67.25^{b}	3.98^{g}	24.39^{b}	24.71^{b}	2.15^{h}	0.68^{c}
	6	(0, ½, ½)	4.35^{b}	9.17^{a}	6.03^{a}	6.26^{a}	68.51^{c}	2.00^{c}	25.29^{b}	25.37^{b}	2.44^{i}	0.71^{c}
	7	(⅓,⅓,⅓)	4.22^{b}	9.17^{a}	7.05^{ab}	6.12^{a}	66.88^{a}	3.72^{f}	23.15^{b}	23.44^{b}	2.44^{i}	0.74^{c}
	Control	-	4.42^{c}	9.83^{c}	7.54^{ab}	7.06^{b}	81.43^{d}	7.14^{h}	21.74^{b}	22.88^{b}	0.25^{a}	0.77^{c}

[1]Mean values (N = 4) with different letters in the same column are significantly different; $P \leq 0.05$.
x_1: strawberry; x_2: raspberry; x_3: "pitanga"; St: *S. thermophilus*; Lb: *L. bulgaricus*; La: *L. acidophilus*;
L*: lightness; a*: redness; b*: yellowness; c*: Chroma; K: Consistency index; N: Flow behaviour index

Contour plots obtained for EA contents reinforced the significant influence of fruits pulp addition to the yoghurt bases (Figure 1). It can be noticed that the center of the triangles represent the optimum mixture compositions of the three fruit pulps that increase EA contents. Any deviation of this point would allow a mixture composition, resulting in a decrease of EA content, for both storage times.

The results of this study indicated that the addition of red berry pulps enhanced EA amounts in symbiotic yoghurts, which could potentially increase their beneficial effect to the host. Given the low number of human studies concerning EA (Landete, 2011; Larrosa et al., 2010), as well as the controversy observed in some of the current results, *in vitro* and *in vivo* studies with a significant number of volunteers are essential in order to evaluate the bioavailability and the effect of inter-individual variability on the levels of EA metabolites detected in the plasma. This would allow and to associate their consume with the beneficial human effects improvement.

Table 2: Coefficients of the polynomial models for the response variables.
ns: not significant; d2: 36 h after preparation; d21: after 21 days of cold storage.

Factors	d2	d21
Strawberry (β_1)	3.896	5.065
Raspberry (β_2)	1.448	1.027
Pitanga (β_3)	1.621	1.119
Strawberry + Raspberry (β_{12})	1.271	6.828
Strawberry + Pitanga (β_{13})	-3.642	ns
Raspberry + Pitanga (β_{23})	8.362	2.222
Strawberry + Raspberry + Pitanga (β_{123})	-16.692	-40.293
P (model)	0.000	0.000
Adjusted R^2	0.9899	0.9995

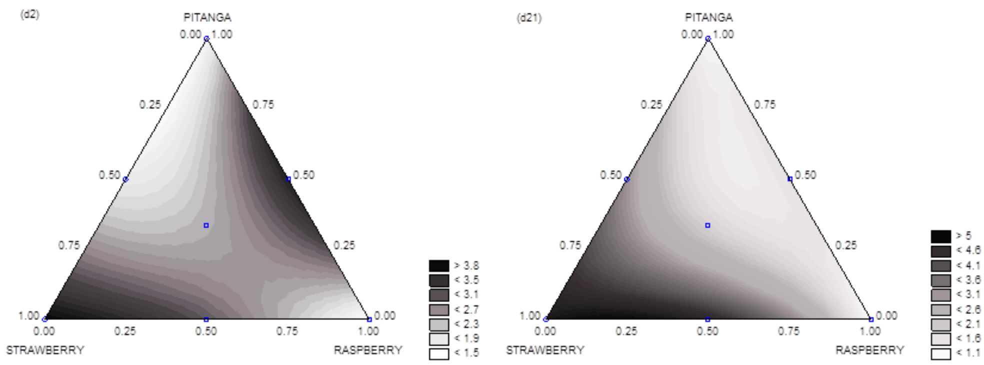

Figure 1: Contour plots showing the effects of strawberry, raspberry and "pitanga" pulps addition on ellagic acid profile (μg.mL^{-1}) after 36 h (d2) of preparation of probiotic yoghurts, and after 21 days of cold storage.

4. Conclusions

Addition of strawberry, raspberry and "pitanga" pulps enhanced the physical properties (colour and rheological) of probiotic yoghurts. Counts of probiotic bacteria slightly decrease during storage, remaining in adequate levels to assure probiotic effects. It was possible to estimate the optimum mixture compositions of the three fruit pulps that increase ellagic acid contents in probiotic yoghurts.

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