French Marine Bark Extract Pycnogenol as a Possible Enrichment Ingredient for Yogurt

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ABSTRACT

The influence of Pycnogenol, French marine bark extract, added to yogurt preparation on the viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* and on pH, titratable acidity, macronutrients, and folate content were evaluated throughout the shelf life of products. At all concentrations studied, Pycnogenol additions neither significantly affected the growth of microorganisms nor caused any modification of nutritional parameters during storage in yogurt. To highlight any possible degradation of Pycnogenol components by yogurt flora, an estimation of total polyphenol contents and an evaluation of some phenolic compounds in yogurt at the greatest concentration of Pycnogenol were carried out at the beginning and at the end of the study. Our data indicates that neither total polyphenol content nor selected phenolic substances (catechin, epicatechins, chlorogenic acid, and caffeic acid) was affected during the shelf life. In conclusion, these results suggest Pycnogenol as a valuable ingredient to enrich yogurt preparation.

Key words: Pycnogenol, yogurt, polyphenol

INTRODUCTION

There is an increasing interest by consumers in food items recognized as beneficial for health, with regard to food that can be considered of greater nutritional value, low in fats, and rich in bioactive compounds scientifically recognized to be negatively associated with disease risk (Roberfroid, 2007; Waijers et al., 2007). Ideally this food should be appealing, taste good, be low in price and, most importantly for the consumers' choice, should contain all-natural ingredients. Moreover, due to the changes in our nutritional habits (hectic habits, lifestyle, etc.) these characteristics should be combined in a food that is easy to eat. Fermented milk products, and in particular yogurts, also considered to be probiotic, represent a useful class of foods that are

attracting greater interest with increasingly demanding and informed consumers (Roberfroid, 2007).

New formulations of these food items are currently present in the food market, claiming different health benefits and presenting different physical and sensory characteristics—such as drinkable yogurts, probiotic yogurts, low-fat yogurts, and enriched yogurts—often offered to the consumer as original complex mixtures of microorganisms (Isleten and Karagul-Yuceer, 2006) designed to combine probiotic effects of bacteria with healthy positive effects of added substances such as polyphenols, coenzymes, or phytosterols. Several yogurt-based products are marketed with the addition of either fruit or vegetables rich in bioactive food ingredients claimed to have beneficial effects on human health (e.g., papayas, grapes, or tomatoes).

The rationale behind these enrichments is that the ease of consumption of yogurt may improve body health status by maintaining a favorable intestinal microbial profile, possibly lowering cholesterol, and at the same time provide an optimal intake of bioactive components, often referred to as beneficial because of their antioxidant capacities (Sanders, 2003). This policy matches the high expectations of consumers and in turn encourages the consumption of fermented milks.

The standardized extract from the bark of the French maritime pine is one of the most-used herbal-sourced food supplements in the world. It is patented under the brand name Pycnogenol (Horphag Research Ltd., Geneva, Switzerland). Pycnogenol, obtained by water extraction of the bark of the *Pinus pinaster*, has a unique and complex chemical composition which has not been completely elucidated. Its main constituents are known to be phenolic compounds, broadly divided into monomers (catechin, epicatechin, and taxifolin) and condensed flavonoids classified as procyanidins/proanthocyanidins. Pycnogenol also contains a significant amount of phenolic acids (such as caffeic, ferulic, and *p*-hydroxybenzoic acids) and glycosylation products, such as glucopyranosyl derivatives of either flavanols or phenolic acids as minute constituents (Rohdewald, 2002).

Pycnogenol has been reported to have strong antioxidant activity both in vitro and in vivo either in experi-

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mental animals or in humans, and participates in the cellular antioxidant network. Further beneficial effects such as vasorelaxation, immunomodulatory function, and anti-inflammatory activities have been reported, confirming the potential of this extract as an effective phytochemical (Rohdewald, 2002). Clinical trials demonstrated that effective doses of Pycnogenol, giving protective effects on health, range from 50 to 200 mg per day (Hosseini et al., 2001; Farid et al., 2007; Zibadi et al., 2008). Lower doses of Pycnogenol are commonly added in dietary supplements, cosmetic products, and combination and functional foods worldwide. A new yogurt formulation based on enrichment with Pycnogenol could be an expedient way to improve the quality of this food item, with appealing results for consumers increasingly aware of the health claims of foods.

The objective of this study was to evaluate the possibility of formulating a new yogurt product with the addition of Pycnogenol. For this purpose, the effects of different concentrations of Pycnogenol in low fat yogurt on microorganism viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* throughout the shelf life of the yogurt were evaluated along with macronutrient profile, folate content, and some physical characteristics (pH and titratable acidity).

To verify any possible utilization of Pycnogenol compounds by yogurt bacteria during the shelf life of the yogurt, the degradation of some Pycnogenol components during the shelf life of the enriched yogurt was also tested. Total polyphenol contents and catechin, epicatechin, and ferulic acid contents were assessed, among the main phenolic components in pine bark extract (Jerez et al., 2006).

This study was based on yogurt made from milk fermented using *Lb. bulgaricus* and *S. thermophilus*, as this is the simplest microbiological system and represents the basic yogurt culture for many other probiotic dairy products all over the world (Lourens-Hatting and Viljoen, 2001). In fact, even though the market is already suggesting a variety of new products with probiotic properties, the simplest yogurt preparation containing *Lb. bulgaricus* and *S. thermophilus* should also be considered a product with probiotic characteristics because of their proven health benefits for the host (Guarner et al., 2005). Low-fat yogurt was chosen on the basis of its increasing consumption during recent years.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). Standard folic acid, gallic acid, chlorogenic acid, caffeic acid, ferulic

acid, (+)-catechin, and (–)-epicatechin were purchased from Sigma (St. Louis, MO). Eluents, HPLC grade, were obtained from Merck. All chemicals, where not otherwise specified, were obtained from Sigma. Pycnogenol (Lot no. F/1200) was kindly provided by Horphag Research (UK) Limited.

Yogurt Sampling

For each experiment, samples of a low fat yogurt (0.5% fat) of the same batch were purchased directly from the farm (Fattoria Latte Sano, Rome, Italy) on the day of production (shelf life = 40 d) to be sure that all the yogurt samples were maintained with the same storage condition. Yogurts were transferred in a refrigerated cooler bag to the laboratory and then stored at 4°C. This yogurt was made by inoculating skim milk with a mixed culture of *Lb. bulgaricus* and *S. thermophilus*, according to Italian regulation.

Experimental Design

Preliminary tests of viable colonies of *Lb. bulgaricus* and *S. thermophilus* in 10 yogurt samples were carried out before starting the experiments, to identify the range of dilutions giving 30 to 300 colonies/plate, and then facilitate the following microbiological counts. An aliquot of yogurt was also dyed with methylene blue (6 g per L of ethanol) for count confirmation.

On the day of each experiment, a solution 0.9% of Pycnogenol dissolved in distilled sterilized water by heating at 60°C, was prepared and the pH of Pycnogenol solution tested at 3.16. This figure is slightly lower than mean pH value measured in control yogurt samples (about 4.00).

Aliquots of 120 g of yogurt were aseptically dispensed into sterilized glass bottles and 3 different Pycnogenol solutions were added, chosen to achieve final concentration of 10, 20, and 40 mg of Pycnogenol per 125 g of yogurt (a standard serving), named **Pyc1**, **Pyc2**, and **Pyc3**, respectively. Pycnogenol concentrations were added to the chilled fermented yogurts resulting in a Swiss-style yogurt (ingredients added after fermentation). These Pycnogenol concentrations, chosen in agreement with the company, were small enough to not cause significant sensorial modifications in the yogurt, but large enough to potentially give possible beneficial effects in the final products. Two series of control samples, **C** (yogurt as consumed) and **Cw** (yogurt + sterilized water), followed the same protocol as samples with added Pycnogenol. The content of each sample group is reported in Table 1. On the first day (T_0), analysis was carried out after Pycnogenol addition and after mixing the preparation. The routine was repeated

Table 1. Additions of Pycnogenol in yogurt samples

Samples (n)	Yogurt (g)	Pycnogenol solution (mL) + water (mL)	Final weight (g)	Pycnogenol content (mg per 125 g of yogurt)
C (2)	120	0 + 0	120	0
Cw (2)	120	0 + 5.00	125	0
Pyc1 (2)	120	1.15 + 3.85	125	10
Pyc2 (2)	120	2.30 + 2.70	125	20
Pyc3 (2)	120	4.60 + 0.40	125	40

(n) = number of samples for each experiment.

in duplicate on the same day for T_0 , T_3 , T_7 , T_{15} , and T_{30} samples, representing analysis of yogurt samples after 0, 3, 7, 15, and 30 d of Pycnogenol addition, respectively. T_{30} overlaps with the expiry day of the yogurt.

After Pycnogenol addition, yogurt samples were uniformly mixed and stored at 4°C. At each sampling time, yogurts were removed from storage, and for each sample 10 mL was aseptically withdrawn after shaking, and dispensed in a sterile stomacher bag containing 90 mL sterile peptone water (Oxoid Ltd., Basingstoke, UK). Each sample was stomached for 30 s (Stomacher 400, PBI International, Milan, Italy) and diluted for plate counting. Aliquots (1 mL) of each dilution were spread plated in duplicate on MRS agar and on M17 agar for microbiological counts of *Lb. bulgaricus* and *S. thermophilus*, respectively.

After the procedure for microbiological analysis, yogurt samples were subjected to physicochemical and nutritional analysis as described in the appropriate sections. At T_0 and T_{30} days, control samples and yogurts with added Pycnogenol were taken to provide an evaluation of polyphenol profiles and contents. The experiment was repeated 3 times with the same scheme.

Enumeration of Viable Bacteria in Yogurt

Official standardized methods (FIL-IDF, 1997) were used to count the microorganisms of yogurt: *Lb. bulgaricus* using MRS agar (Oxoid Ltd.) adjusted to pH 5.4 and anaerobic incubation at 37°C for 72 h. M17 agar (Oxoid Ltd.) and aerobic incubation at 37°C for 48 h were used for selective enumeration of *S. thermophilus*. These standard media are accepted by the International Dairy Federation for differential enumeration of the 2 yogurt species (FIL-IDF, 1997).

The total aerobic mesophilic microorganism counts were determined on plate count agar (Oxoid Ltd.) following the pour plate method by incubation at 30°C for 72 h. The streptococci and lactobacilli identified on the basis of colony type were confirmed by microscopic examination using a Zeiss KF2 light microscope (Karl Zeiss AG, Göttingen, Germany). To further confirm our analysis, random isolates from suitable plates were picked, purified and tested for Gram stain (Harrigan

and McCance, 1976) and catalase activity (Cowan, 1974).

Chemical and Physicochemical Analyses

Protein, lipid, ash contents, and titratable acidity of each sample at every sampling point were measured according to the official methods (AOAC, 2002). The pH of yogurt samples was measured at room temperature with a calibrated pH probe using a pHM82 standard pH meter (Radiometer, Copenhagen, Denmark) according to Marshall (1992). The pH meter was calibrated using reference pH 4.0 and 7.0 buffer solutions.

Folate Content

At each time sampling, folate in yogurts was extracted by trienzyme treatment as described by Johnston et al. (2001) in the presence of hog kidney conjugase (Phillips and Wright, 1983). Folate content was determined by *Lactobacillus casei* (ATCC 7469, NCIMB Ltd., Aberdeen, UK) microbiological assay according to Wright et al., (2000) using folic acid (F 7876, Sigma) as a standard. Folate concentration in yogurt samples was calculated after the building of a regression plot.

Analysis of Polyphenolic Compounds

At the beginning (T_0) and at the end (T_{30}) of the experimental protocol, total polyphenols in Pyc3 samples (40 mg of Pycnogenol per 125 g of yogurt) were assayed according to Singleton and Rossi (1965), using gallic acid as a standard. In the same samples, chlorogenic acid, caffeic acid, ferulic acid, (+)-catechin, and (–)-epicatechin were measured according to Napolitano et al. (2004). Briefly, compounds were separated through a Luna 10m Phenyl-Hexyl (250 × 4.6 mm) column (Phenomenex), using a Hewlett Packard 1100 HPLC (Analytical Division, Waldbronn, Germany) coupled with a diode array detection. Peak identification was based on retention times by spiking with purified standards. Polyphenols quantification was carried out using external standard calibration curves.

Table 2. Viable counts of *Lactobacillus bulgaricus* in yogurt samples (log₁₀ cfu per g of yogurt)

Time ¹	Sample ²				
	C	Cw	Pyc1	Pyc2	Pyc3
T ₀	6.31 ± 0.09 ^a	6.33 ± 0.20 ^a	6.32 ± 0.24	6.38 ± 0.16	6.34 ± 0.20
T ₃	6.28 ± 0.12 ^a	6.20 ± 0.12	6.26 ± 0.10	6.25 ± 0.09	6.24 ± 0.06
T ₇	6.10 ± 0.09	6.16 ± 0.01	6.08 ± 0.11	6.17 ± 0.18	6.08 ± 0.14
T ₁₅	5.95 ± 0.53	5.88 ± 0.34	5.94 ± 0.36	5.96 ± 0.67	5.97 ± 0.52
T ₃₀	5.49 ± 0.11 ^b	5.72 ± 0.11 ^b	5.73 ± 0.22	5.64 ± 0.14	5.78 ± 0.11

^{a,b}Values with different letters within each column differ significantly ($P < 0.05$).

¹T₀, T₃, T₇, T₁₅, T₃₀ = days after Pycnogenol addition.

²C: control; Cw: control with added water; Pyc1: 10 mg of Pycnogenol per 125 g of yogurt; Pyc2: 20 mg of Pycnogenol per 125 g of yogurt; Pyc3: 40 mg of Pycnogenol per 125 g of yogurt.

Statistical Analysis

After enumerating, bacterial populations from the 3 experiments were converted to log₁₀ colony-forming units value (log₁₀ cfu per g of yogurt). SPSS Statistical Software 12.1 (2003 version, SPSS Inc., Chicago, IL) was used for all statistical analyses. The significance of differences between treatments and between experimental times of storage was assessed by means of Bonferroni comparison test. Differences were considered significant at a level of $P < 0.05$.

RESULTS

The first aim of this study was to investigate the possible effects of the Pycnogenol addition to yogurt on lactic acid bacteria viability throughout the product's shelf life. Torras et al. (2005) reported that low doses of Pycnogenol have an antimicrobial activity against different pathogenic microorganisms. However, the effect of Pycnogenol on viability and/or on metabolism of lactic acid bacteria has not yet been investigated. Table 2 reports the mean viable counts of *Lb. bulgaricus* in controls (C, Cw) and in yogurt supplemented with increasing concentrations of Pycnogenol (Pyc1, Pyc2, Pyc3) upon storage at 4°C up to 30 d after addition.

Viability of *Lb. bulgaricus* gradually decreased in the course of the storage period in a similar fashion in all yogurt series (controls and yogurt + Pycnogenol). After 30 d of storage, there was less than 1-log₁₀ cycle difference in the number of *Lb. bulgaricus* with respect to T₀ in controls and in yogurt with different concentrations of Pycnogenol. Our data are in agreement with previously reported observations indicating that a storage effect on *Lb. bulgaricus* viability occurs for lactic acid bacteria in yogurt during shelf life (Birollo et al., 2000). The decrease in bacterial viability was unaffected by the presence of Pycnogenol. In fact, no significant differences ($P \geq 0.05$) in *Lb. bulgaricus* counts were

observed between controls and the 3 different doses of Pycnogenol at any sampling time.

Table 3 reports the trend of *S. thermophilus* viability in yogurts with and without Pycnogenol. *Streptococcus thermophilus* count was larger (2 cycles log₁₀ more) than that of *Lb. bulgaricus*. In all samples we observed counts in the order of 8 log₁₀ cfu per g of yogurt at T₀. Viable cell counts remained stable throughout the storage period (T₃₀). Similarly to *Lb. bulgaricus*, *S. thermophilus* viability counts in yogurt were not affected by the presence of Pycnogenol at any of the considered concentrations. Both *Lb. bulgaricus* and *S. thermophilus* in Pyc1, Pyc2, and Pyc3 remained stable during the shelf life of the products. Plate count agar counts (data not shown) confirmed this data: microscopic examinations showed the presence of streptococci and lactobacilli and total viable counts in yogurt added with Pycnogenol are about 8 log₁₀ cfu per g of yogurt in all the samples, at the expiry date of yogurt.

pH values and titratable acidity of yogurt samples monitored throughout shelf life are shown in Figure 1 and Figure 2, respectively. pH values were not affected by storage with respect to control samples at any sampling times and no significant differences ($P \geq 0.05$) were observed between yogurt with and without Pycnogenol. The initial pH at T₀ ranged from 4.00 in C to 4.07 in Pyc3 and did not change significantly at the end of the experimental window. All yogurt samples showed similar titratable acidity (see Figure 2). No significant differences ($P \geq 0.05$) in acidity were observed between controls and samples with added Pycnogenol, with the exception of yogurt with the greatest concentration of Pycnogenol (Pyc 3 at T₀).

Sugar and protein content in controls and samples with different Pycnogenol concentrations at each time sampling are reported in Figure 3 and Figure 4, respectively. Sugar content, mainly lactose, in yogurt (about 89%), was the same for controls and samples with Pycnogenol (Figure 3), confirming that enrichment

with this polyphenol-rich extract did not promote any fermentative activities of the lactic acid bacteria. Similarly, protein content was not affected by Pycnogenol addition in yogurt (see Figure 4) throughout the experimental period. Lipid content in all yogurts samples ranged from 0.09 to 0.11 g per 100 g of yogurt, and was not affected by either the addition of Pycnogenol or the storage time. The mineral content, expressed as ash, ranged between 0.75 and 0.80 g per 100 g of yogurt in all yogurt samples, remained unchanged throughout the experimental period, and was not affected by Pycnogenol addition.

Folate stability in yogurt samples during storage at 4°C is reported in Table 4. Folate was very stable throughout the shelf life both in controls and in yogurt with Pycnogenol, maintaining levels of about 10 µg per 100 g of yogurt. Folate levels in this conventional yogurt containing *Lb. bulgaricus* and *S. thermophilus*, were not particularly great compared with other probiotic combinations (Crittenden et al., 2003; S. Ruggeri, unpublished data), but this bacterial combination provides good stability in terms of folate content during the shelf life of this product. Moreover, the incorporation of different amounts of Pycnogenol in conventional yogurt did not affect bacterial metabolism in these 2 bacteria species, and folate concentration remained unchanged.

Maintaining the characteristics of the food item is a goal when planning food fortification. In the yogurt formulation with Pycnogenol considered in the present study, microbiological, nutritional, and chemical properties of yogurt have been maintained throughout the shelf life. However, it is also equally important to preserve the bioactive substances and in particular the phenolic component in Pycnogenol. In fact, if significant degradation or metabolization occurs during shelf life by lactic acid bacteria, the use of this extract as an ingredient in fermented milk could be seriously limited.

Table 5 reports an estimation of total polyphenols content and of selected phenolic compounds at the 2

Figure 1. pH in controls and in yogurts with different concentrations of Pycnogenol during shelf-life. C: control; CW: control with added water; Pyc1: 10 mg of Pycnogenol per 125 g of yogurt; Pyc2: 20 mg of Pycnogenol per 125 g of yogurt; Pyc3: 40 mg of Pycnogenol per 125 g of yogurt.

extreme time points of the study (T₀ and T₃₀) in Pyc3, samples; that is, the sample with the greatest concentration of Pycnogenol. Total polyphenols content, as determined by Folin-Ciocalteu method, was not different between the start and the end of the study and a quite large value of total polyphenols were detected (about 27 mg per 100 g of yogurt as gallic acid equivalent) in comparison with other enriched yogurt available in the market. According to our HPLC analysis, epicatechin is the main monomeric phenolic compound (6.67 mg per 100 g of yogurt), followed by catechin (2.41 mg per 100 g of yogurt) in Pyc3 samples at the start of the experiments. The phenolic acids chlorogenic acid and caffeic acid, were detected at lower concentrations (0.39 and 0.24 mg per 100 g of yogurt, respectively) in the same samples.

Table 3. Viable counts of *Streptococcus thermophilus* in yogurt samples (log₁₀ cfu per g of yogurt)

Time ¹	Sample ²				
	C	Cw	Pyc1	Pyc2	Pyc3
T ₀	8.69 ± 0.08	8.78 ± 0.12	8.64 ± 0.15	8.78 ± 0.00	8.61 ± 0.06 ^a
T ₃	8.65 ± 0.16	8.73 ± 0.13	8.69 ± 0.17	8.71 ± 0.11	8.73 ± 0.07
T ₇	8.77 ± 0.16	8.73 ± 0.15	8.73 ± 0.18	8.75 ± 0.13	8.82 ± 0.06
T ₁₅	8.82 ± 0.10	8.90 ± 0.16	8.83 ± 0.08	8.85 ± 0.05	8.86 ± 0.12
T ₃₀	8.83 ± 0.01	8.81 ± 0.07	8.82 ± 0.12	8.79 ± 0.08	8.77 ± 0.10 ^b

^{a,b}Values with different letters within each column differ significantly ($P < 0.05$).

¹T₀, T₃, T₇, T₁₅, T₃₀ = days after Pycnogenol addition.

²C: control; Cw: control with added water; Pyc1: 10 mg of Pycnogenol per 125 g of yogurt; Pyc2: 20 mg of Pycnogenol per 125 g of yogurt; Pyc3: 40 mg of Pycnogenol per 125 g of yogurt.

Figure 2. Titratable acidity (g of lactic acid per 100 g) in controls and in yogurts with different concentrations of Pycnogenol during shelf-life. C: control; Cw: control with added water; Pyc1: 10 mg of Pycnogenol per 125 g of yogurt; Pyc2: 20 mg of Pycnogenol per 125 g of yogurt; Pyc3: 40 mg of Pycnogenol per 125 g of yogurt.

DISCUSSION

This study addressed the effect of Pycnogenol addition on bacterial growth and composition in yogurt throughout the shelf-life within a range of concentrations suitable for commercial use.

The majority of available data on the beneficial effects of Pycnogenol supplementation refer to clinical trials in which large dosages (100 or more mg/d) have been used (Hosseini et al., 2001; Farid et al., 2007; Zibadi et al., 2008). In this study, lower doses of Pycnogenol were used for the new yogurt formulations (10, 20, and 40 mg of Pycnogenol per 125-g serving); in designing a food fortification, lower doses of the bioactive ingredient should be planned, considering daily and long-term consumption, cost/benefits, and other factors (i.e., taste).

Microbiological studies indicate that the bioactive substances in Pycnogenol, which presents a specific distinctive phenolic profile not recognizable in other food extracts, do not affect the growth of *Lb. bulgaricus* and *S. thermophilus* in yogurt as observed in the literature for other ingredients (Ramchandran and Shah, 2008). In particular, phenolic mixtures of different component profiles and concentrations, have been reported to significantly affect the growth of probiotics, when added to yogurt (Öztürk and Öner, 1999; Awaisheh et al., 2005).

Awaisheh et al. (2005) showed a slight decline in the count of *S. thermophilus* and *Lb. bulgaricus* and an increase of *B. infantis* viability in yogurt containing isoflavones, phytosterols and n-3 fatty acids. Concen-

trated grape juice added to yogurt lead to a significant decrease of starter bacteria, probably due to the inhibitory effect of hydroxymethyl furfural (Öztürk and Öner, 1999). On the contrary, other studies addressed more complex food recipes of “mixed berry + yogurt” and “passion fruit + yogurt” reporting no effect on *Lactobacillus acidophilus* and *Bifidobacterium animalis* viability with respect to plain yogurt throughout the shelf life (Kailasapathy et al., 2008).

Our results clearly indicate that the bioactive substances in Pycnogenol do not affect the growth rate of *Lb. bulgaricus* and *S. thermophilus* in yogurt. Also, under our experimental conditions, streptococci appear to be more stable than *Lb. bulgaricus* throughout the shelf-life of the products, as a positive consociation effect between these 2 yogurt bacteria (Altieri et al., 2008).

Total viable counts in yogurt with added Pycnogenol satisfy the “minimum therapeutic dose” (Gill and Prasad, 2008), being about 10^8 through the shelf-life and allowing beneficial effects on intestinal flora. This study also demonstrated that Pycnogenol addition to yogurt, within a range of concentrations suitable for commercial use, does not alter most important chemical parameters and nutrient profile in yogurt throughout the shelf life. The stability of pH, acidity, and sugar contents in yogurt with Pycnogenol indicates that its properties, including the antioxidant capacity, do not lead to significant changes in the metabolism of a basic yogurt culture of *Lb. bulgaricus* and *S. thermophilus*.

Previously reported observations (Kneifel et al., 1993; Donkor et al., 2005) indicate that the most important contributing factor to the loss of cell viability is decreased pH occurring during storage, caused by the accumulation of organic acids as the result of growth

Figure 3. Sugar content in yogurt samples during shelf-life. C: control; Cw: control with added water; Pyc1: 10 mg of Pycnogenol per 125 g of yogurt; Pyc2: 20 mg of Pycnogenol per 125 g of yogurt; Pyc3: 40 mg of Pycnogenol per 125 g of yogurt.

Table 4. Folate content in yogurt samples during shelf-life ($\mu\text{g}/100\text{ g}$ of yogurt)

Time ¹	Sample ²				
	C	Cw	Pyc1	Pyc2	Pyc3
T ₀	10.3 $\pm$ 1.38	10.2 $\pm$ 0.90	9.9 $\pm$ 0.31	9.7 $\pm$ 2.18	10.4 $\pm$ 1.14
T ₃	10.7 $\pm$ 1.02	10.3 $\pm$ 0.54	10.3 $\pm$ 0.40	10.8 $\pm$ 0.96	10.0 $\pm$ 1.09
T ₇	11.2 $\pm$ 0.94	11.3 $\pm$ 0.28	10.0 $\pm$ 0.62	10.8 $\pm$ 0.91	10.7 $\pm$ 0.80
T ₁₅	10.8 $\pm$ 0.55	10.9 $\pm$ 0.60	10.2 $\pm$ 1.00	10.7 $\pm$ 0.38	10.4 $\pm$ 0.92
T ₃₀	10.5 $\pm$ 0.91	11.0 $\pm$ 0.84	10.7 $\pm$ 0.95	11.1 $\pm$ 0.48	10.2 $\pm$ 1.10

¹T₀, T₃, T₇, T₁₅, T₃₀ = days after Pycnogenol addition.

²C: control; Cw: control with added water; Pyc1: 10 mg of Pycnogenol per 125 g of yogurt; Pyc2: 20 mg of Pycnogenol per 125 g of yogurt; Pyc3: 40 mg of Pycnogenol per 125 g of yogurt.

and metabolic activity. The differences in titratable acidity observed between controls and samples with the highest concentration of Pycnogenol (Pyc 3 at T₀) is probably caused by the effect of procyanidins contained in the extract, which was eventually buffered by living cells.

The stability of macronutrient content (especially sugars) and of folate content in yogurt after Pycnogenol additions throughout the shelf life confirms that Pycnogenol components do not substantially alter lactic acid metabolism during the shelf life of yogurt. In fact, among the vitamin B group, folate has been reported to be very sensitive to changes in lactic acid metabolism. Some lactic acid bacteria species require folate for growth and other bacterial strains are capable of synthesizing it (Lin and Young, 2000; Crittenden et al., 2003). Because greater folate intake is desirable in relation to its role in preventing important diseases (DeWals et al., 2007; Mischoulon and Raab, 2007; Sanderson et al., 2007), it would be interesting to design a new functional food with the combination of selected probiotic bacteria producing greater folate

content and Pycnogenol addition. This natural folate enrichment should reinforce Pycnogenol and probiotic beneficial effects on human health.

Studies on phenolic components of Pycnogenol showed that they remain quite stable in yogurt during shelf life. The phenolic profile of Pycnogenol is complex and not yet completely defined. Complete qualitative analysis has not been carried out (Packer et al., 1999; Jerez et al., 2006). This scarcity of data causes difficulty in comparing our results with previously published data. Virgili et al. (2000) reported data on ferulic acid, caffeic acid, and p-coumaric acid content in Pycnogenol. Their reported value for caffeic acid content in Pycnogenol was lower than that determined in the present work. Probably, the HPLC method used here provided better quantification of phenolic compounds.

Our results encourage further analysis on phenolic compounds contents in Pycnogenol when used to enrich yogurt, and future studies for the evaluation of their in vivo bioavailability. Further studies on contents and bioavailability/bioaccessibility of the main minerals, calcium and phosphorus, in yogurt fortified with Pycnogenol should also be necessary to verify possible effects of its polyphenol substances in limiting or promoting mineral intestinal absorption. The stability of microbiological, chemical, and nutritional parameters

Figure 4. Protein content in yogurt samples during shelf-life. C: control; Cw: control with added water; Pyc1: 10 mg of Pycnogenol per 125 g of yogurt; Pyc2: 20 mg of Pycnogenol per 125 g of yogurt; Pyc3: 40 mg of Pycnogenol per 125 g of yogurt.

Table 5. Total polyphenol contents and amount of phenolic compounds in Pyc3 samples¹ at the start and the end of experiments

Phenolic compound	T ₀ ^{2,3}	T ₃₀ ^{2,3}
Total polyphenols (by Folin-Ciocalteu) ⁴	27.01 $\pm$ 1.31	26.9 $\pm$ 1.11
Catechin (by HPLC)	2.41 $\pm$ 0.291	2.05 $\pm$ 0.256
Chlorogenic acid (by HPLC)	0.39 $\pm$ 0.046	0.46 $\pm$ 0.058
Epicatechin (by HPLC)	6.67 $\pm$ 0.641	6.31 $\pm$ 0.702
Caffeic acid (by HPLC)	0.24 $\pm$ 0.025	0.21 $\pm$ 0.023
Total (by HPLC)	9.80	9.03

¹40 mg of Pycnogenol per 125 g of yogurt.

²T₀, T₃₀ = days after Pycnogenol addition.

³All values expressed as mg per 100 g of yogurt.

⁴Expressed as gallic acid equivalents.

along with the stability of phenolic substances, suggest Pycnogenol as a very good candidate for yogurt fortification.

Obviously, to promote these new products it will be also fundamental to investigate sensorial properties as color, flavor, and other physicochemical parameters such as viscosity, degree of syneresis, as well as biochemical and proteolytic activities of yogurt microorganism such as angiotensin-I-converting enzyme (ACE), or α -glucosidase activities. Moreover, further studies are warranted in vivo for the evaluation of the effects of the low doses of Pycnogenol intake with yogurt on the antioxidant status and on different biological outcomes in humans such as platelet aggregation and anti-inflammatory activity.

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