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Development of Milk Fermented with *Lactobacillus acidophilus* Fortified with *Vitis vinifera* Marc Flour

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Summary

Some by-products of wine industry still contain nutrients and functional compounds that make them potential ingredients to formulate new high value-added food products. The aim of this study is to develop milk fermented with *Lactobacillus acidophilus* fortified with marc flour of different cultivars of *Vitis vinifera* from wine production and to evaluate their influence on fermentation kinetics, probiotic counts, phenolic compounds, sugar content and antioxidant activity. The acidification time was significantly shortened by these enrichments (by up to 2.7 h), and the bacterial count during cold storage resulted in stronger fortification of samples (up to 4.13 %) when compared to control tests. Fermented milk containing grape marc showed considerable amounts of phenolic compounds with notable antioxidant activity, as well as significant amounts of total sugars. The most important aspect of this paper is the feasibility of using winery by-products, rich in phenolic compounds, as natural supplements to fortify probiotic-fermented milk.

Key words: fermented milk, grape marc, *Lactobacillus acidophilus*, phenolic compounds

Introduction

Top 40 wine-producing countries generate more than 26 million of tonnes of wine per year (*1*) along with a huge amount of by-products called grape marc, which is thrown away or used as a base for distillate production (*2*). However, such a process leads to a large amount of waste material that can be used as a fuel (*3*), contributing to the reduction of carbon dioxide emissions. Grape marc is composed of proteins, fibres (*4*) and considerable amounts of functional substances such as phenolic compounds (malic, tartaric and succinic acids, tannins and anthocyanins) (*5–9*), prebiotics (fructooligosaccharides), fatty acids (linoleic and mainly conjugated linoleic acids) and nutrients, which can be used as ingredients for the formulation of new functional foods (*10*).

Free radicals play a key role in several human diseases, for instance atherosclerosis, arthritis, Alzheimer's and Parkinson's diseases, tumour promotion and carcinogenesis. Antioxidants are good scavengers of free radicals and can be employed as inhibitors of neoplastic processes. A large number of synthetic and natural antioxidants have been demonstrated to induce beneficial effects on human health and disease prevention. This capacity has been attributed to wine because of its considerable content of polyphenols, which are well-known antioxidant compounds (*11*) with recognized anti-inflammatory properties (*12*).

Various methods based on combinations of parameters were investigated to extract polyphenols from grape marc (*5–7*). For instance, conventional extraction was assessed by Casazza *et al.* (*6*) varying the extraction time and solid/liquid ratio, and the combined effects of temperature and pressure were investigated using subcritical water extraction by Aliakbarian *et al.* (*5*).

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Dairy probiotic foods represent a large part of the market of functional foods (*13*). According to Food and Agriculture Organization (FAO) (*14*), probiotics can be defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host. Many species of the genus *Lactobacillus* have been used as probiotics and, amongst them, the strains belonging to the *Lactobacillus acidophilus* species stand out for their probiotic activity, proven through clinical trials (*15*).

Although in literature some reports on foods enriched with polyphenolic grape extracts can be found (*16–19*), there is none on fermented milk developed using pure grape marc. In light of this, the aim of this study is the development of a new probiotic-fermented milk product fortified with the addition of fermented or non-fermented wine marc. Whole grape marc samples were used to establish a cost-effective methodology able to reduce the overall time of the extraction step. Milk bases were fermented by a strain of *L. acidophilus*, and the influence of different grape marc samples on total fermentation time, probiotic counts during two weeks of cold storage, polyphenol and sugar contents and antioxidant activity of the resulting dairy products were investigated.

Materials and Methods

Grape marc samples

Fermented and non-fermented marc of Croatina (FCM and NFCM, respectively), Freisa (FFM and NFFM) and Timorasso (FTM and NFTM) cultivars were kindly provided by a wine producer from Tortona (Alessandria, Italy) and stored at 20 °C. Marc samples were separated from stalks, dried at 50 °C for 72 h and then reduced in a grinder (Moulinex, Paris, France) to fine powder with particle size <600 μm, measured through sieves (this granulometry was adopted to give a smooth consistence to the product). Powders were placed in sealed bags and stored protected from the light at 4 °C.

Fermentation

Skimmed milk powder (fat 2 g/L; Naturei®, Cavazzoli, Italy) was reconstituted to 120 g/L in distilled water. Marc samples were added separately at 10, 20 and 50 g/L final concentrations to reconstituted milk samples, and one sample without grape marc was kept as control. Next, the milk bases were heat-treated at 85 °C for 15 min under agitation in a water bath, model Y14 (Grant Instruments, Cambridge, UK), then divided into sterile Falcon tubes (50 mL), brought to 42 °C in a thermostatic bath and then inoculated with 1 mL of a *Lactobacillus acidophilus* (strain NCFM) suspension. Fermentations were carried out in water bath at 42 °C, and the fermentation kinetics was followed through the pH decrease by a pH-meter, model pH211 (Hanna Instruments, Woonsocket, RI, USA), within a period of 24 h to evaluate the fermentation profiles in the presence or absence of the selected grape marc samples. The pH was checked every 60 min until pH=5.0 was reached, and then every 15 min until the end of fermentation (pH=4.7). After that, the samples were immersed in an ice bath and cooled to 15 °C, then the gels of fermented milk samples were broken by mild agitation and stored in tubes at 4 °C until further analyses.

Microbiological analyses

L. acidophilus counts were performed, either with FCM or NFCM, after 1, 7 and 14 days of cold storage at 4 °C. Serial dilutions of 1 mL of each sample with 9 mL of sterile peptone water (1.0 g/L) were prepared. Afterwards, the dilutions were plated in MRS agar, pH=6.2 (Difco, Franklin Lakes, NJ, USA) using the pour plate technique (*20*) and incubated at 37 °C for 72 h in a jar under anaerobic conditions obtained with AnaeroGen sachets (Oxoid, Ogdensburg, NY, USA). Bacterial concentration was expressed as log CFU per mL of fermented milk.

Total phenolic compound and sugar determination

Methanolic extraction was carried out according to Revilla *et al.* (*21*) with some modifications. Briefly, 1.0 g of each sample (fermented milk and fibres) was placed in 100 g of a water/methanol solution (80 mL of methanol in 100 mL of water) and then stirred for 2 h at room temperature. The Folin-Ciocalteu method (*22*) was used to determine the concentration of total phenolic compounds in milk, fermented milk and fibres. Each sample was filtered under vacuum, the liquid fraction was transferred to an acrylic cuvette and the absorbance was measured at 765 nm by an UV/Vis spectrophotometer, model Lambda 25 (PerkinElmer, Wellesley, MA, USA). To prepare the calibration curve, gallic acid (Sigma-Aldrich, St. Louis, MO, USA) was used as standard, at concentrations from 0 to 300 μg/mL in deionised water. The total phenolic content was expressed in mg of gallic acid equivalents (GAE) per g of milk. The analyses were performed in triplicate on samples submitted to cold storage for 1 day.

Total carbohydrate content of methanolic extracts from fermented milk and fibres was determined according to the method described by DuBois *et al.* (*23*). Briefly, 200 μL of sample were mixed in a test tube with 200 μL of 40 g/L phenol solution (Merck, Darmstadt, Germany) and 1.0 mL of concentrated sulphuric acid solution (96 mL of sulphuric acid in 100 mL of water; Carlo Erba, Milan, Italy). After agitation in a vortex, the reaction tube was cooled in an ice bath for 30 min, and the resulting solution was analysed by the above spectrophotometer at 490 nm. Lactose (Sigma-Aldrich) was used as standard to prepare the calibration curve (0.062 to 1.0 mg/mL), while 200 μL of deionised water were used instead of sample solution to prepare the control.

Antioxidant activity

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method was used to measure the antioxidant activity of the fermented milk and fibres as described by Re *et al.* (*24*). For this purpose, potassium persulphate (Sigma-Aldrich) was added to a 7-mM ABTS solution (Sigma-Aldrich) to form the radical cation $ABTS^+$. The antioxidant activity was measured as the capacity of the sample to reduce $ABTS^+$ to ABTS. The aqueous solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Sigma-Aldrich) was used as antioxidant standard to prepare a calibration curve with the concentration varying from 0 to 1.0 g/L.

Statistical data processing

All the analyses and trials were carried out in triplicate, with the exception of those of bacterial counts, which were made in quadruplicate. The influence of various parameters was assessed by the analysis of variance (ANOVA), and the Tukey's *post hoc* test was used for mean discrimination or mean comparison, depending on circumstances. Multiple comparison of the means was performed by least significant difference (LSD) test ($p \leq 0.05$). The statistically significant differences were illustrated by different letters in tables and figures. The STATISTICA software v. 8.0 (StatSoft, Tulsa, OK, USA) was used for analyses.

Results and Discussion

Cultivar selection

Different fermented or non-fermented grape marc samples of Croatina (FCM, NFCM), Freisa (FFM, NFFM) and Timorasso (FTM, NFTM) cultivars were selected and characterized in terms of moisture, total phenolic compounds, total carbohydrates and antioxidant activity (Table 1). Among them, NFCM had the highest phenolic content and antioxidant activity, followed by FCM. The non-fermented grape marc from all three cultivars contained more carbohydrates compared to the fermented ones. Statistical differences were observed for the moisture content of grape marc samples ($p \leq 0.05$).

Marc variety and its concentration in the milk base (10, 20 or 50 g/L) were chosen on the basis of either its ability to lower pH at the end of 24-hour fermentation compared to the initial value or of its phenolic content (Fig. 1). The milk fortified with FFM showed a percentage of pH decrease >35 % at all tested grape marc concentrations, an aspect that could be considered industrially desirable in terms of reduction of time needed to complete the fermentation (pH=4.70). However, as shown in Table 1, the phenolic content of this residue as GAE ((20.0±0.2) mg/g) was undesirably lower ($p \leq 0.05$) than that of FCM and NFCM ones. Moreover, FFM caused a massive milk protein precipitation in all samples already during the thermal treatment phase.

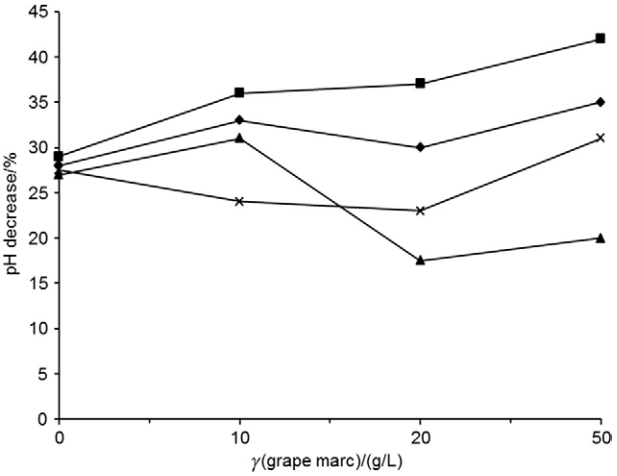

Fig. 1. Percentage of pH decrease with respect to initial pH=6.6 at the end of 24-hour fermentations of milk fortified or not with different grape marc samples at different concentrations. (◆) FTM (fermented Timorasso marc), (■) FFM (fermented Freisa marc), (▲) FCM (fermented Croatina marc), (×) NFCM (non-fermented Croatina marc). Data are expressed as the mean values of three analyses

The milk fortified with FTM had inferior characteristics to the FFM for it exhibited a smoother pH decrease after the fermentation (by 30–35 %), and, besides that, its polyphenolic content was still remarkably lower than that of FCM or NFCM ($p \leq 0.05$).

On the other hand, FCM was the best fortifying agent, in that it promoted a percentage decrease in pH after the milk fermentation by only 23 %. In addition, the phenolic content of this grape marc was notably greater than that of the other cultivars. NFCM showed an acidification profile similar to FCM (percentage pH decrease by 26 %), with comparable polyphenolic content. For these reasons, both Croatina marc samples were selected as milk supplements in the subsequent studies of the fermentation process and fermented milk characterization from the product quality viewpoint. To this purpose, fractions of both grape marc samples were fixed at 2 % to ensure a satisfactory value added to the product and a smooth consistence.

Table 1. Physicochemical characteristics of grape marc

Fibre type	*w*(total carbohydrates) / mg/g	*w*(TP as GAE) / mg/g	Antioxidant activity as TE / mg/g	Moisture / %
FCM	(122.6±0.7)^{b}	(46.2±0.5)^{e}	(620.9±5.6)^{d}	(55.46±0.04)^{a}
NFCM	(240.5±0.7)^{f}	(48.9±0.7)^{f}	(745.8±4.8)^{e}	(61.13±0.03)^{c}
FTM	(141.8±0.3)^{d}	(18.5±0.3)^{a}	(527.5±5.5)^{c}	(66.54±0.05)^{e}
NFTM	(160.8±0.2)^{e}	(29.2±0.4)^{c}	(564.5±5.6)^{a}	(69.38±0.04)^{f}
FFM	(96.1±0.3)^{a}	(20.0±0.2)^{b}	(466.9±10.3)^{b}	(55.81±0.02)^{b}
NFFM	(134.1±0.8)^{c}	(37.6±0.1)^{d}	(554.2±5.6)^{a}	(63.05±0.04)^{d}

TP=total phenolics, GAE=gallic acid equivalent, TE=Trolox equivalent, FCM=fermented Croatina marc, NFCM=non-fermented Croatina marc, FTM=fermented Timorasso marc, NFTM=non-fermented Timorasso marc, FFM=fermented Freisa marc, NFFM=non-fermented Freisa marc. For each treatment, mean values in a column followed by different letters in superscript (from a to f) are significantly different ($p \leq 0.05$). Values are means±standard deviations of three replicates

Fermentation kinetics

It can be observed in Fig. 2 that milk enriched with FCM and NFCM exhibited different acidification profiles, especially in terms of time needed to complete the fermentation (pH=4.7), notwithstanding an almost coincidental starting pH=6.6. As expected, the fermentation of the control milk without marc took the longest (14.5 h), while the addition of both Croatina marc samples significantly ($p \leq 0.05$) shortened this time (up to 11.8 h with FCM and 12.8 h with NFCM).

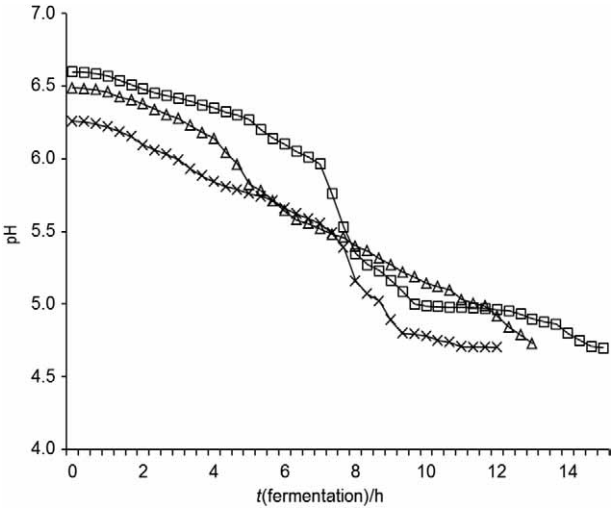

Fig. 2. pH decrease during the fermentation of: (□) control milk, (×) milk enriched with fermented Croatina marc, and (△) milk enriched with non-fermented Croatina marc

Considering the whole fermentation process, the mean acidification rate of the control, FCM- and NFCM-fermented milk was $2.2 \cdot 10^{-3}$, $2.2 \cdot 10^{-3}$ and $2.3 \cdot 10^{-3}$ pH_{units}/min, respectively. Even though these mean values did not show any statistical difference among them ($p>0.05$), those of the maximum acidification (V_{max}) did, being $9.2 \cdot 10^{-3}$, $22 \cdot 10^{-3}$ and $11 \cdot 10^{-3}$ pH_{units}/min, respectively. While the fermentation of NFCM milk showed no marked variations of the acidification profile along the fermentation, with a peak of $1.4 \cdot 10^{-3}$ pH_{units}/min after 7.8 h, the control and FCM-fermented milk samples exhibited two noticeable accelerations, with peaks of $4.4 \cdot 10^{-3}$ and $5.2 \cdot 10^{-3}$ pH_{units}/ min, respectively (Fig. 2).

These results point out different patterns of *L. acidophilus* adaptation to the different milk bases and suggest that FCM may contain compounds able to stimulate lactic acid production by this probiotic. *L. acidophilus* has in fact been proven to consume preferentially fructose from the fruit rather than lactose from the milk (*25*); besides, the wine marc likely provided extra sugar content to the fermentation media. These two factors can explain the shorter fermentation time in milk samples containing marc compared to the control. Moreover, the wine fermentation of Croatina grape may have converted a portion of fructooligosaccharides into more easily fermentable mono- and disaccharides available for *L. acidophilus* metabolism, hence resulting in faster fermentation of FCM- rather than of NFCM-fermented milk.

L. acidophilus grew remarkably throughout the fermentation in fortified milk. After 1 day of cold storage following fermentation, the bacterial count, which was 10.47 log CFU/mL in the control, did in fact increase to 10.81 and 10.69 log CFU/mL in samples containing FCM and NFCM, respectively. In spite of the well-known antibacterial activity of phenolic compounds present in wine marc, they did not exert any statistically significant influence ($p>0.05$) on probiotic counts in both fermented fortified milk samples during storage (Fig. 3). Nonetheless, this finding is in accordance with previous observations (*26–28*).

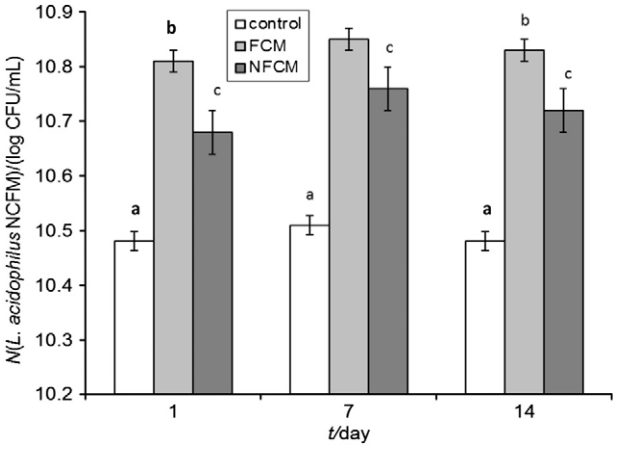

Fig. 3. Viable counts of *Lactobacillus acidophilus* in fermented control milk and milk enriched with fermented (FCM) and non-fermented Croatina marc (NFCM), after 1, 7 and 14 days of storage at 4 °C. Error bars are standard deviations with respect to the mean values of three analyses

Total sugar content

The mass fraction of total sugars in milk samples was assessed after 1 day of cold storage in order to get information on the nutritional value of fermented products. This value was 9.49, 14.6 and 16.8 mg per 100 g (Fig. 4) in the control, FCM- and NFCM-fermented milk, respec-

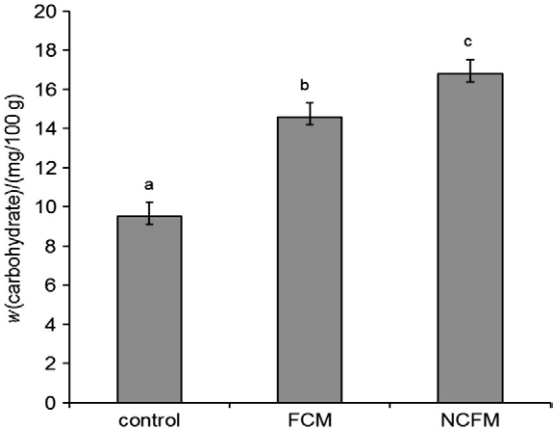

Fig. 4. Carbohydrate mass fraction in fermented control milk and milk enriched with fermented (FCM) and non-fermented Croatina marc (NFCM), after 1 day of storage at 4 °C. Error bars are standard deviations with respect to the mean values of three analyses

tively, which demonstrates that the addition of both marc samples increased significantly ($p \leq 0.05$) the sugar content of the fermented milk as the likely result of the marked presence of sugars in its composition (Table 1).

Antioxidant activity and content of phenolic compounds

As Fig. 5a shows, the mass fraction of phenolic compounds in FCM- and NFCM-fermented milk samples increased by 70.2 and 81.4 % respectively, when compared to the control. The control showed a moderate positivity to Folin-Ciocalteu test probably due to its casein content which exerts a moderate antioxidant activity (*29,30*). Moreover, as expected, the higher the phenolic compound fraction, the higher the antioxidant activity, which increased by about 19 and 37 times in FCM- and NFCM--fermented milk respectively, compared to the control. Although both types of Croatina marc increased the antioxidant activity, the milk containing non-fermented marc exhibited a higher antiradical power than the other (Fig. 5b).

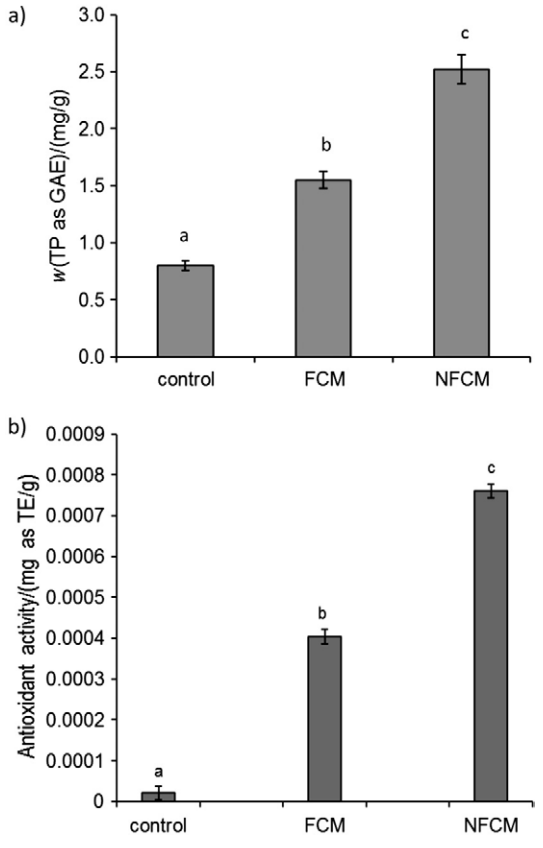

Fig. 5. a) Total polyphenolic (TP) content, and b) antioxidant activity of fermented control milk and milk enriched with fermented (FCM) and non-fermented Croatina marc (NFCM), after 1 day of storage at 4 °C. Error bars are standard deviations with respect to the mean values of three analyses. GAE=gallic acid equivalent, TE=Trolox equivalent

Contrary to the current opinion that phenolic compounds are responsible for inhibition of microbial growth, surprisingly, the amount of phenolic compounds did not influence the probiotic counts in this work. Nonetheless, this observation is in accordance with the findings of Parkar *et al.* (*31*), who reported that polyphenols, some of them present in wine marc, were able to even stimulate the growth of a probiotic strain of *Lactobacillus rhamnosus* along with its adhesion to gut cell lines.

Conclusions

Lactobacillus acidophilus was used to ferment skimmed milk supplemented with Croatina, Freisa and Timorasso marc, and the fermentation kinetics, probiotic counts, phenolic compounds and carbohydrate content and antioxidant activity were evaluated. Both fermented (FCM) and non-fermented (NFCM) Croatina marc samples were better supplements compared to the others. While the fermentation of the control milk did not show any marked variation in the acidification profile, both fortified milk samples, especially the one containing FCM, exhibited remarkable accelerations of pH decrease, likely associated with their sugar contents. The FCM-fermented milk showed a 1.84 % higher bacterial count compared to the NFCM one, probably because of higher sugar metabolization, and both exhibited greater bacterial counts than the control (4.13 and 2.34 %, respectively). Both FCM- and NFCM-fermented milk samples also showed high antioxidant activity associated with the polyphenolic compounds contained in marc, which makes them new dairy products of some potential interest for developing markets interested in new functional foods. It can be said that the use of the grape marc proposed in this paper could provide an alternative way to exploit such a residue, which is currently wasted or burned. The promising results of this work suggest the use of *L. acidophilus* in further studies addressed to this issue.

References

1. Food and Agriculture Organization (FAO) (*http://faostat3.fao.org/home/index.html*).
2. D. Sales, M.J. Valcárcel, L. Pérez, E. Martínez-Ossa, Physical-chemical treatments applied to wine-distillery waste, *Bull. Environ. Contam. Toxicol. 37* (1986) 407–414.
3. A.R. Celma, S. Rojas, F. López-Rodríguez, Waste-to-energy possibilities for industrial olive and grape by-products in Extremadura, *Biomass Bioenergy, 31* (2007) 522–534.
4. L. Zalikarenab, R. Pirmohammadi, A. Teimuriyansari, Chemical composition and digestibility of dried white and red grape pomace for ruminants, *J. Anim. Vet. Adv. 6* (2007) 1107–1111.
5. B. Aliakbarian, A. Fathi, P. Perego, F. Dehghani, Extraction of antioxidants from winery wastes using subcritical water, *J. Supercrit. Fluids, 65* (2012) 18–24.
6. A.A. Casazza, B. Aliakbarian, D. De Faveri, L. Fiori, P. Perego, Antioxidants from winemaking wastes: A study on extraction parameters using response surface methodology, *J. Food Biochem. 36* (2012) 28–37.
7. L. Fiori, D. De Faveri, A.A. Casazza, P. Perego, Grape by--products: Extraction of polyphenolic compounds using supercritical CO_2 and liquid organic solvent – A preliminary investigation, *CyTA-J. Food, 7* (2009) 163–171.
8. A.A. Casazza, B. Aliakbarian, E.Y. Ortiz Montoya, P. Perego, *t*-Resveratrol recovery from grape skins using high pressure and temperature extraction, *J. Biotechnol.* (Suppl.), *150* (2010) 333.

9. A.A. Casazza, B. Aliakbarian, S. Mantegna, G. Cravotto, P. Perego, Extraction of phenolics from *Vitis vinifera* wastes using non conventional techniques, *J. Food Eng. 100* (2010) 50–55.
10. Z.M. Jin, J.J. He, H.Q. Bi, X.Y. Cui, C.Q. Duan, Phenolic compound profiles in berry skins from nine red wine grape cultivars in Northwest China, *Molecules, 14* (2009) 4922–4935.
11. A.M. Alonso, D.A. Guillén, C.G. Barroso, B. Puertas, A. García, Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content, *J. Agric. Food Chem. 50* (2002) 5832–5836.
12. D. Palmieri, B. Pane, C. Barisione, G. Spinella, S. Garibaldi, G. Ghigliotti *et al.*, Resveratrol counteracts systemic and local inflammation involved in early abdominal aortic aneurysm development, *J. Surg. Res. 171* (2011) e237–e246.
13. A.P. do Espírito Santo, P. Perego, A. Converti, M.N. Oliveira, Influence of food matrices on probiotic viability – A review focusing on the fruity bases, *Trends Food Sci. Technol. 22* (2011) 377–385.
14. *Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria*, A joint FAO/WHO expert consultation, FAO (Food and Agriculture Organization) and WHO (World Health Organization), Cordoba, Argentina (2001) p. 12.
15. M.E. Sanders, T.R. Klaenhammer, Invited review. The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic, *J. Dairy Sci. 84* (2001) 319–331.
16. N. Harbourne, J.C. Jacquier, D. O'Riordan, Effects of addition of phenolic compounds on the acid gelation of milk, *Int. Dairy J. 21* (2011) 185–191.
17. M. Karaaslan, M. Ozden, H. Vardin, H. Turkoglu, Phenolic fortification of yogurt using grape and callus extracts, *LWT-Food Sci. Technol. 44* (2011) 1065–1072.
18. J.E. O'Connel, P.F. Fox, Significance and applications of phenolic compounds in the production of milk and dairy products: A review, *Int. Dairy J. 11* (2001) 103–120.
19. A. Rózek, I. Achaerandio, C. Güell, F. López, M. Ferrando, Use of commercial grape phenolic extracts to supplement solid foodstuff, *LWT-Food Sci. Technol. 43* (2010) 623–631.
20. H. Kodaka, S. Mizuochi, H. Teramura, T. Nirazuka, Comparison of the compact dry TC method with the standard pour plate method (AOAC Official Method 966.23) for determining aerobic colony counts in food samples: Performance-tested method, *J. AOAC Int. 88* (2005) 1702–1713.
21. E. Revilla, J.M. Ryan, G. Martín-Ortega, Comparison of several procedures used for the extraction of anthocyanins from red grapes, *J. Agric. Food Chem. 46* (1998) 4592–4597.
22. V.L. Singleton, J.A. Rossi Jr., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Vitic. 16* (1965) 144–158.
23. M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, *Anal. Chem. 28* (1956) 350–356.
24. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorizing assay, *Free Radic. Biol. Med. 26* (1999) 1231–1237.
25. J.W. Nielsen, S.E. Gilliland, The lactose hydrolyzing enzyme from *Lactobacillus acidophilus*, *Cult. Dairy Prod. J. 27* (1992) 20–28.
26. I. García García, J.L. Bonilla Venceslada, P.R. Jiménez Peña, E.R. Gómez, Biodegradation of phenol compounds in vinasse using *Aspergillus terreus* and *Geotrichum candidum*, *Water Res. 31* (1997) 2005–2011.
27. M.A. Bustamante, C. Paredes, R. Moral, J. Moreno-Caselles, A. Pérez-Espinosa, M.D. Perez-Murcia, Uses of winery and distillery effluents in agriculture: Characterization of nutrient and hazardous components, *Water Sci. Technol. 51* (2005) 145–151.
28. R. Devesa-Rey, X. Vecino, J.L. Varela-Alende, M.T. Barral, J.M. Cruz, A.B. Moldes, Valorization of winery waste *vs.* the costs of not recycling, *Waste Manag. 31* (2011) 2327–2335.
29. G. Cervato, R. Cazzola, B. Cestaro, Studies on the antioxidant activity of milk caseins, *Int. J. Food Sci. Nutr. 50* (1999) 291–296.
30. B.J. McGookin, M. Augustin, Antioxidant activity of casein and Maillard reaction products from casein-sugar mixtures, *J. Dairy Res. 58* (1991) 313–320.
31. S.G. Parkar, D.E. Stevenson, M.A. Skinner, The potential influence of fruit polyphenols on colonic microflora and human gut health, *Int. J. Food Microbiol. 124* (2008) 295–298.