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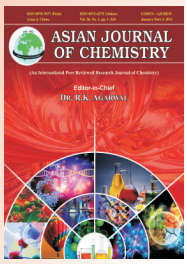

Determination of Organic Acid Composition and Free Radical Scavenging Capacity of Kefir

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The organic acid composition as well as the free radical scavenging capacity of kefir produced from different kinds of milk was investigated. The organic acid composition of the samples was determined by high performance liquid chromatography (HPLC), while free radical scavenging capacity was evaluated spectrophotometrically. Oxalic acid, acetic acid, citric acid and succinic acids were found to be significantly higher in kefir produced from cow milk ($p < 0.05$), while malic acid and lactic acid were significantly higher in kefir produced from goat milk ($p < 0.05$). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of kefir produced from goat milk was significantly higher than that of kefir made from cow milk ($p < 0.05$). There was a high correlation between the malic acid and lactic acid formation and the free radical scavenging activity of the samples, implying that the formation of these organic acids during kefir production positively enhances the free radical scavenging capacity of the fermented milk products.

Keywords: Kefir, Organic acids, HPLC, Free radical scavenging.

INTRODUCTION

Kefir is a traditional fermented milk product, usually produced with a natural starter culture or with a complex starter culture made up of different kinds of lactic acid bacteria, acetic acid bacteria and yeasts[1,2]. Kefir is considered to originate from the Caucasus Mountains. For many years this beverage has been very popular in Turkey, the former Soviet Union, Hungary, Bulgaria and Poland, but nowadays its consumption has also been spread to Sweden, Norway, Finland, Greece, Germany, France, Austria, Portugal, Brazil, Argentina, Israel, Taiwan, India and Australia[2,3]. Kefir might be produced from different types of milk such as cow, goat, sheep, ewe, rice or soy and recently some other substrates are suggested as substrates for kefir production[3-5]. Kefir is known to be not only a good source of nutrients such as carbohydrates, proteins, minerals and vitamins[1,2,6-9], but is also rich in probiotic microorganisms[1-3,6,9], thus leading to kefir's functional health benefits[1-3,9].

Nowadays, the investigation of the functional and antioxidant properties of probiotic products which can protect human body from free radicals and retard the progress of many chronic diseases is one of the most important points of research. Free radicals are usually generated during metabolic reactions in the body[10]. They have been shown to be harmful as they react with important cellular components such as proteins, DNA and cell membrane[11]. The method of determining the radical scavenging activity by using 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) is an established *in vitro* method usually employed for the evaluation of antioxidant activity of natural materials.

The free radical DPPH possesses a characteristic absorption at 515 nm (purple in colour), which decreases significantly on exposure to radical scavengers (by electron donation or by providing hydrogen atoms). A lower absorbance at 515 nm indicates a higher radical scavenging activity of the compound. This test is usually used as a standard assay in antioxidant activity studies[12].

In recent decades, the significance of goat milk as a product with high nutritional value has been noticed and nowadays, goat milk is considered to be healthier than cow milk in having better digestibility, alkalinity, buffering capacity, therapeutic values in medicine and human nutrition[13]. Furthermore, kefir produced from goat milk has been reported to have better antioxidant properties[14] and to be a better mineral source than kefir produced from other types of milk[15].

The organic acids present in fermented dairy products are regarded to be highly effective in the flavor formation of the respective products[16,17]. Furthermore, the organic acids present in fermented milk products such as kefir have been shown to have antimicrobial effects[18] and antimutagenic properties[19].

The main purposes of this study were (i) to analyze comparatively the organic acid profile of kefir produced from cow and goat milk, (ii) to evaluate the free radical scavenging capacity of the samples and (iii) to determine the relationship between the organic acid formation and the free radical scavenging capacity of the samples.

EXPERIMENTAL

Kefir grains were obtained from Eker AS, Bursa, Turkey. Pasteurized cow and goat milk were obtained from Ataturk Orman Ciftligi, Ankara. All reagents used during the analyses were of analytical grade and obtained from Merck (Darmstadt, Germany). All organic acids used as external standards were of HPLC-grade.

Preparation of kefir: Pasteurized cow and goat milk were inoculated with 5 % kefir grains. The cow and goat milk samples were incubated at 25 °C until pH of the fermented liquid reached 4.6. Afterwards, in order to remove the kefir grains, the samples were filtered through three layers of cheesecloth. The kefirs from cow and goat milk were manufactured separately in triplicate. The samples were then immediately prepared for analysis.

Determination of the organic acids: At the end of the fermentation 10 mL of samples were added to 40 mL 0.02 M H_2SO_4, vortexed and centrifuged at 10 000 g for 10 min. For organic acid determination, the resulting supernatants were further filtered through 0.45 µm membrane filter (Millipore). The simultaneous determination of oxalic acid, malic acid, lactic acid, acetic acid, citric acid and succinic acid using liquid chromatography was carried out according to Arnetoli *et al.*[20]. The chromatography analysis was carried out using a HPLC system (Shimadzu, Japan). The equipment of the HPLC system consisted of LC-20AD pump, SIL-20A Auto sampler, SPD-20A Prominence UV/visible detector, DG4-20AS prominence degasser and LC solution (version: 1.23 sp1) software. An Inertsil ODS-III C18 column (46 × 150 ID, 5 µm particle size) was used for the chromatographic separation. The mobile phase was carried out with 125 mM KH_2PO_4 adjusted to pH 2.5 with *o*-phosphoric acid. The flow rate of the mobile phase was adjusted at 1 mL/min. The wavelengths of the UV detection were performed at 210 nm for oxalic acid, malic acid, lactic acid, acetic acid, citric acid and succinic acid.

The retention times of each organic acid preparing single standard solution at 50 mg L^{-1} concentrations were determined before calibration with a mix solution of all organic acids for simultaneous determination. The standard mix solution of organic acids was prepared by using oxalic acid, malic acid, lactic acid, acetic acid, citric acid and succinic acid, with ultrahigh-grade quality water (Milli-Q Reagent Grade Water System, Millipore). Then, the equipment was calibrated with a mix solution of the organic acids at different concentrations. The typical reference spectra of the organic acids are given in Fig. 1. Unless otherwise stated, all measurements of the organic acids of the samples were done in triplicate.

DPPH free radical scavenging activity assay: The effect of the oxidized kefir extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) was estimated as described by Brand-Williams *et al.*[21]. Immediately after fermentation, 5 g of samples was added to

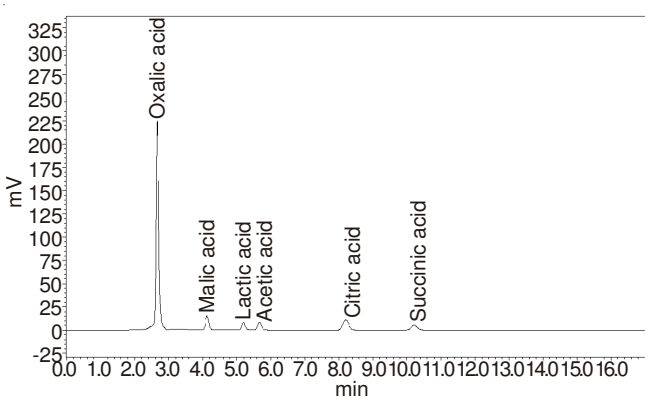

Fig. 1. Organic acid spectrum (50 mg L^{-1})

25 mL 0.02 M H_2SO_4, vortexed and centrifuged at 10 000 g for 10 min. The resulting supernatants were used for free radical scavenging activity determination. The DPPH solution was added to the diluted sample, thoroughly mixed, then incubated for 0.5 h for the reaction to occur. Afterwards, the absorbance of the sample was measured at 515 nm using a UV-visible spectrophotometer (Thermo Aquamate). The absorbance of DPPH solution in methanol, without any antioxidant (control), was also measured. The percentage of DPPH radical scavenging activity was calculated by using the following equation:

$$\% \text{ DPPH scavenging} = [(A_{control}-A_{sample})/A_{control}] \times 100$$

where A_{sample} is the absorbance of the sample after the time necessary to reach the plateau (0.5 h) and $A_{control}$ is the absorbance of DPPH. Extract concentrations providing IC_{50} inhibition values (defined as the concentration of the compounds that was able to inhibit 50 % of the total DPPH radicals) were calculated from graph plotting using nonlinear regression and expressed in microgram material equivalents per milliliter for sample extracts. Butylated hydroxytoluene (BHT) and α-tocopherol was used as a positive control. A lower value of IC_{50} indicates a higher antioxidant activity and *vice versa*.

Statistical analysis: The results were reported as mean ± SD (standard deviation). T-test was used to determine the differences between means of organic acids and one-way ANOVA was applied to investigate the differences among means of the values for free radical scavenging determination by using Statghaphics Centurion XV software. The values were considered to be significantly different at $p < 0.05$. The correlation coefficients (r) were calculated in order to determine the relationship between the organic acid formation and the free radical scavenging capacity using MS Excel software.

RESULTS AND DISCUSSION

Fermented dairy products have been consumed for years worldwide. The functional properties as well as the health benefits of fermented dairy products have been partly ascribed to the higher antioxidant properties of these products. For this reason, the free radical scavenging capacity and the organic acid content of kefir from different types of milk was determined in this study.

The formation of organic acid during fermentation is the result of the hydrolysis of fatty acids (namely butterfat), biochemical metabolic processes as well as microbial metabolism[17].

Furthermore, the presence of organic acids contributes beneficially to the specific flavor and sensory products of dairy products[2,16].

The composition of organic acids of kefir samples in the end of the fermentation is presented in Table-1. Oxalic acid, acetic acid, citric acid and succinic acid were statistically higher in kefir produced from cow milk ($p < 0.05$), while malic and lactic acids were statistically higher in kefir made from goat milk ($p < 0.05$).

TABLE-1
ORGANIC ACID COMPOSITION OF KEFIR PRODUCED FROM COW MILK AND GOAT MILK*

Organic acids	Cow milk Kefir (mg L^{-1})	Goat milk Kefir (mg L^{-1})
Oxalic acid**	169.15 ± 1.16[a]	119.37 ± 0.74[b]
Malic acid	145.65 ± 2.71[a]	3082.93 ± 6.61[b]
Lactic acid	12695.65 ± 25.69[a]	17641.90 ± 40.54[b]
Acetic acid	11848.55 ± 36.20[a]	3831.64 ± 12.24[b]
Citric acid	762.24 ± 2.01[a]	25.44 ± 0.46[b]
Succinic acid	485.63 ± 3.29[a]	264.03 ± 1.90[b]

*The results are given as Mean ± Standard Deviation
**Values within a row with different letters are significantly different ($p < 0.05$)

The concentrations of organic acids in fermented milk mainly vary with the type of milk, the type and composition of the starter culture and the duration of fermentation. Lactic acid and acetic acid compositions were previously determined in varying amounts[4-6,17]. Garrote *et al.*[6] suggested that the formation of lactic acid and acetic acid was dependent on different kefir grains types. Seydim-Guzel *et al.*[17] found lower lactic acid, higher citric acid in kefir produced from cow milk and no acetic acid was detected in kefir after 24 h of fermentation. Gronnevik *et al.*[22] analyzed the chemical properties of kefir during storage and found out that citrate content decreased, while lactate and acetate concentrations increased during the first week of storage, while in the next weeks no significant changes occurred. In our study, the concentration of acetic acid was found to be significantly higher in kefir made from cow milk, implying the relatively high metabolic activities of the acetic acid bacteria present in kefir grains.

Hydroxyl radicals are regarded to be the most harmful reactive oxygen species (ROS) that are responsible for the oxidative injury of biomolecules[23]. DPPH is a stable free radical, readily reacting with proton radical scavengers. Therefore, the DPPH free-radical scavenging method is thoroughly used in the evaluation of the antioxidant capacity of various materials[12,24]. α-Tocopherol and BHT were used as positive controls in the comparative analysis of the free radical scavenging capacity of kefir produced from cow and goat milk. The IC_{50} values of kefir made from cow and goat milk were determined as 188.35 and 105.12 mg/mL, respectively (Table-2). On the other hand, % inhibition results of kefir produced from cow milk and goat milk were found to be 52.84 and 71.96, respectively (Fig. 2). As can be seen from Table-2, kefir made from goat milk has a significantly higher radical scavenging capacity than kefir produced from cow milk ($p < 0.05$). Similarly, Liu *et al.*[14], demonstrated that kefir produced from goat milk exhibited higher antioxidant capacity.

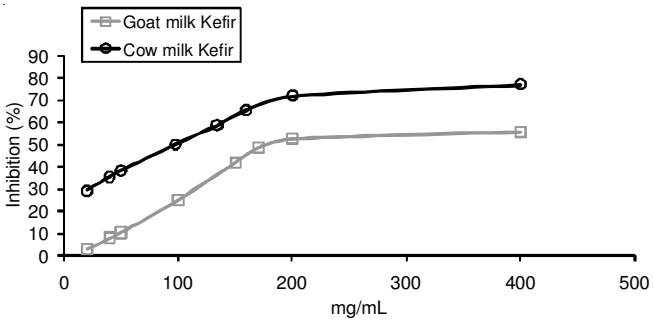

Fig. 2. Scavenging effect of kefir samples on 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The organic acids formed as the result of the microbial metabolism in fermented products are known to have significant antimicrobial properties[18]. Furthermore, acetic acid and lactic acid have been determined to exhibit anitmutagenic properties[19]. Nevertheless, some organic acids like acetic, malic and citric acid have been found to enhance the free radical scavenging activity of ascorbic acid[25]. In the present study, there was a high correlation between % inhibition level of DPPH and the lactic acid ($r = 0.997$) and malic acid content ($r = 0.998$). This correlation is in conjunction with the results of Scalzo[25] and the conclusions of Lankaputhra and Shah[19] about the synergistic free radical scavenging and antimutagenic properties of organic acids.

Conclusion

Dairy products are not only significant for human nutrition, in terms of their protein, fat and mineral content, but they have also beneficial health effects. In this study, the free radical scavenging capacity, as well as the organic acid composition of kefir made from goat and cow milk was investigated comparatively. Malic acid and lactic acid were found to be higher in kefir made from goat milk and also there was a positive correlation between the malic acid and lactic acid formation and the free radical scavenging capacity of the samples. It can be concluded that, malic acid and lactic acid formation in fermented dairy products positively enhance the free radical scavenging capacity of these products and also that fermented products made from goat milk have better antioxidant properties.

TABLE-2
DPPH FREE RADICAL-SCAVENGING ACTIVITY ASSAY*

	Cow Milk Kefir	Goat Milk Kefir	BHT	α-Tocopherol
IC_{50} (mg/mL)**	188.35 ± 1.21[a]	105.12 ± 1.02[b]	0.033 ± 0.01[c]	0.043 ± 0.03[c]
Inhibition (%)	52.84 ± 0.15[a]	71.96 ± 0.79[b]	96.84 ± 0.16[c]	98.36 ± 0.44[c]

*The results are given as Mean ± Standard Deviation
**Values within a row with different letters are significantly different ($p < 0.05$)

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