FLAVONOIDS AND TERPENOIDS IN GOAT MILK IN RELATION TO FORAGE INTAKE

FLAVONOIDI E TERPENOIDI NEL LATTE DI CAPRA IN RELAZIONE AL FORAGGIO

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<th>ABSTRACT</th>
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<td>Milk from goats fed borage or hawthorn shows the presence of flavonoids and terpenoids. A comparison between the flavonoid content in the plants and in the milk of the goats seems to prove that the mammary route is one of the excretory pathways. Rutin and quercetin are excreted in part without modifications, while other compounds appear to have less structural complexity, indicating a metabolism that is probably mediated by the gastrointestinal microflora. The presence of phenolic...</td>
<td>Il latte di capre alimentate con borragine o biancospino rivela la presenza di flavonoidi e terpenoidi provenienti dalla dieta. Il confronto fra il contenuto flavonoidico delle piante e del latte degli animali studiati sembra provare che l'escrezione mammaria sia una delle vie metaboliche possibili per i composti flavonoidici. La rutina e la quercetina sono parzialmente escrete senza modificazioni, mentre altri composti presenti nel latte presentano minore complessità strutturale rispetto ai me...</td>
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- Key Words: Borago officinalis, Crataegus oxyacantha, flavonoids, goat milk, mammary excretion -
compounds in the milk could affect the quality and sensory traits of the milk and milk products.

tabolici presenti nelle piante, a riprova di una probabile mediazione metabolica da parte delle microflora gastrointestinali. La presenza di composti fenolici nel latte può essere importante in relazione alla qualità sensoriale del latte e dei suoi derivati.

INTRODUCTION

Mediterranean pastures in southern Italy are highly variable depending on the season. The proportion of graminaceous plants decreases from 80-85% to 55-60% from winter to spring, while the proportion of forbs increases from 25-30% to 60-65%, from late spring to early summer (FEDELE et al., 1993). In early summer, goats graze predominantly on forbs (FEDELE et al., 1993), some of which are used as medicinal plants by man.

In recent years, several studies have shown that some plant compounds (e.g., vitamins, volatile organic compounds) are directly transferred from the grazed herbage to the milk (FEDELE et al., 2000; PIZZOFERRATO et al., 2000a; VIALLON et al., 2000). In fact, the physico-chemical and organoleptic features of milk and cheese can be greatly affected by the diet of the grazing animals since a large variety of the organic components present in the plant tissues can accumulate in the lipid and water-soluble fraction of the milk. In particular, monoterpenic compounds can greatly influence the aroma of the milk. Terpenic compounds could be used as biochemical indicators of the composition of the forage grazed and could be used to determine the origin of cheese (FEDELE et al., 2000).

The impact of animal diet on the monoterpenic and sesquiterpenic content in cheese has been studied by many authors. DUMONT and ADDA (1978), DUMONT et al. (1981), BOSSERT et al. (1994, 1997, 1999) and BUCHIN et al. (1998) have shown that these aromatic compounds are more frequent in dairy products when animals are fed mountain forages. Other authors (VIALLON et al., 1999; COULON et al., 2000; VERDIER-METZ et al., 2000) have reported that highland grass with different botanical composition strongly affects the flavour of milk and cheese.

Little information is available on the fate of other non-volatile phyto-constituents and the possibility of finding these compounds and/or their metabolites in milk. O'CONNEL and FOX (2001) reported that the majority of phenolic compounds found in cow milk are derived from the feed and when the cows are fed large quantities of particular crops, other phenolic compounds may also be detected in ruminant milk. Moreover, some alkylphenols present in ruminant milk are derived from phenolics ingested through the animal feed (KILIC and LINDSAY, 2005).

The aim of this study was to investigate the effect of some secondary metabolites of two plants (Crataegus oxyacantha L. and Borago officinalis L.), whose chemical composition is well known (Zhang et al., 2001; Peiretti et al., 2004), on the chemical composition of milk. These plants are normally preferred for grazing by goats in the spring and summer (FEDELE et al., 1993).
MATERIALS AND METHODS

Plant material

Borage (Borago officinalis L.) and hawthorn (Crataegus oxyacantha L.) were collected at the Bella farm of the Istituto Sperimentale per la Zootecnia, Potenza, Italy. The plants were identified using PIGNATTI's "Flora d'Italia" (1976). A voucher specimen of the plants is stored in the Herbarium of the Medical Botany Chair at the State University of Salerno.

Animal experiment

Nine lactating Maltese goats were divided into three equal groups and each group was housed in a box (3x2 m). Group C (control) was fed ad libitum with a diet of natural hay ad libitum and 300 g/per head day~1 of concentrates (barley 200 g and maize 100 g).

Groups H (hawthorn) and B (borage) were fed ad libitum with green plants of C. oxyacantha and B. officinalis, respectively. A sufficient quantity of borage and hawthorn in the bud-flowering stage was harvested daily from a native pasture on the farm of the Istituto Sperimentale per la Zootecnia. No vitamins, minerals or concentrates were added to diet of these animals.

During ten days of adaptation to the diet, the feeding behaviour was evaluated. Thereafter, data were collected regarding the measurements of daily plant intake and milk production for each group for seven days. Cumulative milk samples for each group were collected daily and stored in bottles at -20°C.

Plant samples made up of the plant parts usually grazed by the goats were harvested from the same pasture. The samples were air dried and preserved in canvas sacks until used for chemical analysis.

Plant extraction and analysis

Five-hundred grams of air-dried borage stems, flower buds and flowers were successively extracted with petroleum ether, chloroform and methanol at room temperature at a concentration of 10% w/v to give 2.2, 2.8 and 3.7 g of residues, respectively. Air-dried leaves, flowers and terminal sprouts of hawthorn (500 g) were extracted in the same way, obtaining 2.6 g of petroleum ether, 4.7 of chloroform and 5.8 g of methanol residues.

Both chloroform extracts were fractionated on a silica-gel (Merck, Milan, Italy) column (80x4 cm) eluting with chloroform and mixtures of CHCl₃ and MeOH of increasing polarity.

Fractionation of the borage chloroform extract gave 467 fractions that were pooled into 17 main fractions (B1-BXVII) on the basis of their thin layer chromatography (TLC) similarity. The hawthorn chloroform extract was fractionated into 576 fractions, and then pooled into 15 main fractions (H1-HXV).

Fractions containing flavonoid compounds (BVII and HVIII) were purified by RP-HPLC on a μ-Bondapack column (30 cm x 7.8 mm) eluting with a 1:1 mixture of H₂O-MeOH.

Quercetin (12.3 mg) and 5,7-dihydroxyflavone (3.5 mg) were recovered from fraction BVII. Fraction BIX yielded pure oleanolic acid (2.5 mg).

Fraction HVIII afforded pure quercetin (12.7 mg) and vitexin-7-glucoside (2.5 mg).

Methanol extracts of borage and hawthorn were both fractionated in the same way. Methanol extracts were fractionated in a H₂O-BuOH system. BuOH extracts dissolved in methanol were purified on a Sephadex LH-20 (Amersham, Milan, Italy) column, eluting with MeOH.

Ninety-four fractions were obtained from the borage methanol extracts (BME), which were then grouped into 8 main fractions (BME1-BMEVIII) on the basis of their TLC similarity. Fraction BMEV was purified by RP-HPLC under the same conditions as reported above; rutin (4.5 mg), kaempferol (4.6 mg) and
kaempferol 3-O-β-D-glucopyranoside (1.9 mg) were recovered.

Hawthorn methanol extract (HME) gave 87 fractions, that were pooled into 8 main fractions (HMEI-HMEVIII) on the basis of their TLC similarity in BuOH: AcOH:H₂O (12:3:5) and CHCl₃:MeOH: H₂O (70:30:3). Rutin (8.9 mg) and apigenin (2.3 mg) were obtained as pure constituents by purifying the HMEVI fraction by means of RP-HPLC, on a μ-Bondapack column (30 cm x 7.8 mm) eluting with a 1:1 mixture of H₂O-MeOH.

Five-hundred grams of the diet fed to group C (Control) were extracted and fractionated as reported for borage and hawthorn; no flavonoids were found.

Milk extraction and analysis

One litre of each milk (D₂₀ = 1.021) sample was lyophilized and the residue was extracted successively at room temperature and at a concentration of 10% w/v with petroleum ether, chloroform, methanol. Milk from group H gave 8.9 g, 465.3 mg and 2.6 g of petroleum ether, chloroform and methanol residues, respectively. The milk from group B gave 9.9 g of petroleum ether, 768.9 mg of chloroform and 2.8 g of methanol residues. The milk from group C (control) gave 7.5 g, 879.3 mg and 1.8 g of petroleum ether, chloroform and methanol residues, respectively. Like the chloroform plant extracts, the chloroform milk extracts were fractionated on a silica-gel column eluting with chloroform and mixtures of chloroform and methanol of increasing polarity.

Chloroform extract fractions of milk B were pooled into 9 main fractions (MBI-MBXI). Fraction MBVI was further purified by RP-HPLC on a Bondapack column (30 cm x 7.8 mm) eluting with a mixture of H₂O-MeOH 1:1. Pure compounds of 5,7-dihydroxyflavone (1.4 mg), flavone (2.7 mg) and 5,7,4'-tri-hydroxyflavonol (2.7 mg) were recovered from this fraction. Fraction MBIII, purified in the same way, contained pure β-amyrin (1.5 mg). Methanol extract, treated in the same manner as the plant methanol extracts, was fractionated on a Sephadex LH-20 column. Seventy-five fractions were collected and pooled into seven main fractions MBMEI-MBMVEVII. Fraction MBMEIV, purified by RP-HPLC revealed the presence of rutin (1.7 mg).

Fractions of the chloroform extract of milk H were pooled into 10 main fractions (MHI-MHX). Fraction MVH was purified by RP-HPLC, under the same conditions as reported for the H milk, and revealed pure 5,7-dihydroxyflavonol (2.3 mg), flavone (3.5 mg), and quercetin (1.2 mg). Pure β-sitosterol (1.8 mg) was purified from fraction MHI. The methanol extract did not show the presence of flavonoids.

The control milk did not reveal the presence of flavonoid derivatives in the CHCl₃ or MeOH extracts.

Structural determination of compounds

The structure of the pure compounds that were isolated from both plants and milk was determined by accurate analysis of 1H, 13C and 19F DEPT NMR data and by comparison with literature data (AGRAWAL, 1989). NMR spectra were obtained on a Bruker DRX 600 Spectrometer (Bruker, Karlsruhe, Germany). The identification of the flavonoid compounds isolated from the plants and milk was also confirmed, when possible, by HPLC analyses by comparing the retention times with those of standard compounds (Sigma-Aldrich Co., Milan) (FICARRA et al., 1984, 1990).

Statistical analysis

Determinations of compounds from both plants and milk were repeated three times and the data are reported as mols/kg of dry plant or milk, and represent means of these three determinations.
RESULTS AND DISCUSSION

The feeding behaviour and food intake of goats changed in relation to plant species and feeding regimes. The goats preferred to eat the apex of the stems, the flower buds and flowers of borage, while they preferred the leaves, flowers and terminal sprouts of hawthorn. The goats browsed 85.3 and 51.8% of borage and hawthorn, respectively, in relation to the total amount available for the animals.

The level of borage dry matter intake (DMI) varied from 50 to 220 g/head day⁻¹ (115±33 g); for hawthorn it was from 364 to 955 g/head day⁻¹ (516±44). The DMI of the control group was 860 g/head day⁻¹ (755±105).

The metabolites of plant origin (terpenoids and flavonoids) found in the plant and milk, respectively, are listed in Table 1. The triterpenoids are only represented by oleanolic acid, found in the borage chloroform extract and by β-amyrin, present in the milk (chloroform extract) of the goats fed borage. β-sitosterol was isolated in the chloroform extract of group H.

In contrast, flavonoids were present in all the plant and milk extracts. The chloroform extract of milk from goats fed borage contained 5,7,4'-tri-OH-flavonol, 5,7-di-OH-flavone, and flavone; the methanol extract contained rutin. Milk of the hawthorn-fed group contained quercetin, 5,7-di-OH-flavone, and flavone (chloroform extract). Only the methanol extract of milk from goats fed hawthorn did not contain any of these compounds. The control milk did not contain triterpenoid or flavonoid derivatives. No other phenolics were found in the milk. The chemical composition of the milk from each group (B, H, and control) was homogeneous for the entire experiment.

The milk of both groups B and H contained compounds that probably came from the diet; flavonoids and terpenoids, in fact, are metabolites that are wide-

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<th>Extract</th>
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<th>Milk</th>
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<tr>
<td></td>
<td>Borage</td>
<td>Hawthorn</td>
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<tr>
<td>Chloroform</td>
<td>Quercetin 8.1±0.3x10⁴</td>
<td>Quercetin 8.4±0.6x10⁴</td>
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<td></td>
<td>5,7-di-OH-Flavone 2.7±0.3x10²</td>
<td>Vitexin-7-glucoside 0.8±0.1x10³</td>
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<td></td>
<td>Oleanolic acid 1.1±0.1x10⁵</td>
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</tr>
<tr>
<td>Methanol</td>
<td>Rutin 1.5±0.3x10⁵</td>
<td>Rutin 2.9±0.2x10⁴</td>
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<tr>
<td></td>
<td>Kaempferol 2.0±0.2x10³</td>
<td>Apigenin 1.7±0.2x10⁴</td>
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<td></td>
<td>Kaempferol-3-O-β-D-glucopyranoside 0.9±0.2x10⁵</td>
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Table 1 - Plant metabolites found in plants and milk. Data are expressed as mols/1,000 g of dry plant or milk. Data are means of three determinations.
spread in higher plants. The goats in group B ingested about 435 mg/week of total flavonoidic compounds, excreting about 105 mg/week of these compounds in the milk. The ratio 435/105 is similar to that found for rutin, 80 mg ingested/23 mg excreted. The goats of group H showed a greater intake of dry matter and, consequently, had greater quantities of total flavonoids. The presence of flavonoids in the milk is not unexpected, considering that the mammary route is one of the drug excretion pathways. On the other hand, the literature reports the presence of phenolics in ruminant milk that came from the plants ingested by the animals (O’CONNELL and FOX, 2001; KILIC and LINDSAY, 2005) and flavonoid transport by mammalian endothelial cells has been proposed (SCHRAMM et al., 1999).

The concentration of the total flavonoid fraction found in the milk of both groups is low in comparison to the corresponding fractions found in the plants. This finding is in agreement with the literature which indicates that urinary and biliary excretion of flavonoids and/or their metabolites are the main metabolic pathways for such compounds (BABA et al., 1981; SAWAI et al., 1987; HUTCHINS et al., 1995: LEE CHAO et al., 2002). The concentration of flavonoids in plants ranges from 0.8 to 8.4x10^{-6} mols/kg, whereas the concentration recovered in the milk was about 10-times less, ranging from 2.7 to 16x10^{-6} mols/kg.

The qualitative analysis of the flavonoidic fraction present in the milk provided some insights: 1) two flavonoids (rutin and quercetin) are present both in plants and in the milk; however, their concentration in the milk is low if compared with the concentration found in the plants. GEE et al. (2000) and MORAND et al. (2000) reported that rutin is hydrolysed during absorption in the monogastric digestive tract. The presence of rutin and quercetin in milk may constitute a non-hydrolysed aliquot or may indicate a difference between monogastrics and ruminants. 2) Other flavonoids that were purified from the milk were different from those found in each plant, but generally had a chemical structure that was less substituted than that of the plant compounds.

In the literature, controversies abound concerning which form of flavonoids are absorbed (LEE CHAO et al., 2002). Some animal studies have shown that some flavonoids should be considered non-absorbable because they are bound to sugars such as β-glycosides (DI CARLO et al., 1999). On the other hand, other animal models have shown that gastrointestinal metabolism of flavonoids is dependent on the intestinal microflora (GRIFFITHS et al., 1981; BOKKENHEUSER et al., 1987; WINTER et al., 1989; SCHNEIDER et al., 1999; JUSTESEN et al., 2000; LEE CHAO et al., 2002). Our hypothesis is that the gastrointestinal microflora of the goat can structurally modify feed flavonoids through hydrolyses and/or other interactions that result in molecules that are structurally less complex.

The data presented in this study raise new questions: 1) for how long a time can flavonoids be recovered from milk? 2) What is the concentration of such compounds in the milk in relation to time? 3) Does the presence of such compounds in milk have nutraceutical applications that could improve health benefits of milk for humans?

Flavonoids are well known for their biological activities (DI CARLO et al., 1999) and, in some modern strategies for preparing fortified foods, these compounds are added to milk and other food (NINFALI et al., 2002). Our data demonstrate that the presence of phenolics and terpenoids in milk depends on the animal feed. This finding has also been confirmed for other classes of compounds, e.g. tocopherol and retinol (PIZZOFERRATO et al., 2000b) and volatile terpenes (COULON et al., 2000). Moreover, the consumption of phenolic-rich foods can
affect both the animal's health and the sensory aspects of the milk and their products (O'CONNELL and FOX, 2001).

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