Bioavailability of quercetin from its aglycone and its glucorhamnoside rutin in lactating dairy cows after intraduodenal administration

A. Gohlke,* C. J. Ingelmann,* G. Nürnberg,† A. Starke,‡ S. Wolfram,§ and C. C. Metges*¹

*Department of Nutritional Physiology “Oskar Kellner,” and

†Department of Genetics and Biometry, Leibniz Institute for Farm Animal Biology (FBN), 18196 Dummerstorf, Germany

‡Clinic for Internal Medicine, Faculty of Veterinary Medicine, University of Leipzig, 04103 Leipzig, Germany

§Institute of Animal Nutrition & Physiology, Christian-Albrechts-University of Kiel, 24118 Kiel, Germany

ABSTRACT

Because of their health-promoting properties, flavonoids are used in feed supplements for ruminants, although scientific evidence for their efficacy *in vivo* is limited. It has been shown recently that bioavailability of quercetin is low after ruminal administration in cows because of degradation by the ruminal microbiota. It is unknown whether quercetin could be absorbed from the small intestine in ruminants if degradation is prevented; therefore, we investigated the bioavailability of quercetin after duodenal administration in 6 German Holstein cows. On 88 ± 3 d in milk, each cow received equivalent doses of quercetin [9, 18, or 27 mg of quercetin equivalents (QE)/kg of body weight] either as quercetin aglycone (QA) or as its glucorhamnoside rutin (RU). In addition, 2 control studies with duodenal administration of NaCl solution (0.9%) were conducted per cow to examine concentrations of flavonoids in plasma during regular feeding. Blood samples were collected at defined time intervals over a period of 24 h before and after administration of the test compounds. A washout period of 2 d was applied between the runs to avoid possible carryover effects. Concentrations of plasma quercetin aglycone and its metabolites isorhamnetin, tamarixetin, and kaempferol were measured after treatment with glucuronidase/sulfatase by HPLC with fluorescence detection. After administration of RU, levels of plasma quercetin did not increase above baseline, irrespective of dose administered. After duodenal administration of QA, the plasma concentration of QA and its methylated metabolites clearly increased above baseline. The maximal plasma concentrations of total flavonols (about 2 h after application) increased in a dose-dependent manner but showed high interindividual variability (range 368.8 to 983.3 nmol/L at 27 mg of QE/kg of body weight) but peak time did not differ. Preadministration baseline values of total flavonols

were reached again 3 to 4 h after QA administration. The bioavailability of quercetin and its metabolites, as measured by the area under the concentration–time curve, was affected by the quercetin source applied, whereby quercetin from RU was unavailable. Taken together, duodenal administration enhanced bioavailability of QA almost to values previously reported in pigs after oral administration of QA. In contrast to findings in monogastrics or after oral administration in cows, quercetin from RU seems to be unavailable when administered duodenally.

Key words: bioavailability, dairy cow, flavonoid, quercetin

INTRODUCTION

Because public concern regarding the use of pharmaceuticals in livestock farming is increasing, the application of natural agents, such as flavonoids, as a potential means of improving animal health has become more widespread (Waghorn and McNabb, 2003; Rochfort et al., 2008). Currently, several feed additives for cattle containing flavonoids are commercially available (e.g., http://biopro21.en.ec21.com/POLI_Queen_1L--1523628_1523752.html; http://www.provimi.com.gr/media/grape_pp_concentrate_-_eng.pdf; http://www.exquim.com/en_UK/areasdenegocias/nutricionanimal.asp?id=6445&pag=0; <http://www.biomax.org.uk/citrus-bioflavonoids.php>) but independent scientific evaluation of flavonoid effects in ruminants is lacking (Colitti and Stefanon, 2006; Gladine et al., 2007; Cottica et al., 2011; Berger et al., 2012; Balcells et al., accepted). Because all of these products contain mainly crude extracts, it is impossible to determine whether flavonoids or other bioactive compounds are responsible for the observed effects. Quercetin, a frequently investigated flavonoid, is widely distributed in plants and has been shown to exert antiinflammatory and antioxidative effects *in vitro* and affects glucose and lipid metabolism (Hertog et al., 1992; Middleton et al., 2000; Cermak et al., 2003; de Boer et al., 2005; Reinboth et al., 2010). In nature, quercetin is almost always

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¹Corresponding author: metges@fbn-dummerstorf.de

present in glycosylated form (Arts et al., 2004) and one of the most common quercetin glycosides is rutin, in which the disaccharide 6-*o*-L-rhamnosyl-D-glucose is attached to the aglycone. In monogastrics, the bioavailability of quercetin is higher from the aglycone form than from its glucorhamnoside rutin (Hollman et al., 1999; Graefe et al., 2001; Olthof et al., 2000). Although quercetin aglyca pass the intestinal mucosa in the small intestine by passive diffusion (Walgren et al., 1998; Movileanu et al., 2000; Manach et al., 2004), quercetin glycosides are not absorbed until the linkage between aglycone and sugar moiety is cleaved (Arts et al., 2004; Lesser and Wolfram, 2006). Rutin can only be reduced to the aglycone by microbial enzymes and therefore it is absorbed only from the terminal ileum and the large intestine (Manach et al., 1997; Day and Williamson, 2003). Rumen bacteria, such as *Clostridium orbiscindens* or *Eubacterium ramulus*, do not only release the aglycone from quercetin glycosides but are also capable of splitting the scaffold of flavonoids (Blaut et al., 2003; Lin et al., 2003; Schoefer et al., 2003; Labib et al., 2004). Therefore, it is assumed that quercetin is degraded by enzymes of rumen microbiota after oral administration, which would considerably lower its bioavailability in cattle. Indeed, Berger et al. (2012) recently reported that the concentration of quercetin in plasma of cows was very low after administration of quercetin aglycone in the rumen.

A prerequisite for quercetin to exert any effect in ruminants is its systemic availability, about which little is known. It is not known whether quercetin can be absorbed from the small intestine in ruminants, in contrast to monogastrics. Therefore, this study is the first to explore the bioavailability of quercetin from quercetin aglycone and rutin after duodenal administration. The hypothesis of this study was that, in bypassing the rumen, bioavailability of quercetin would be increased considerably, to a level similar to that observed in monogastrics. Thus, the objectives were (1) to compare the pharmacokinetics of quercetin and its metabolites after duodenal administration with bioavailability data in pigs published previously (Ader et al., 2000; Cermak et al., 2003) and after ruminal administration (Berger et al., 2012), and (2) to investigate acute effects of quercetin on DMI, milk yield, and plasma concentrations of glucose and NEFA.

MATERIALS AND METHODS

Animals and Diet

The experimental procedures were carried out in accordance with the German Animal Protection regulations and were approved by the relevant authorities

of the State Mecklenburg-Vorpommern, Germany (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischereiwesen Mecklenburg-Vorpommern, Germany).

Six German-Holstein cows (>10,000 kg of milk in first lactation) with an average BW of 598.3 ± 27.6 kg were purchased from a local farm (Ahrenshagen, Germany) and fitted with duodenal cannulas in wk 4 of their second lactation. After surgery and during the complete experimental period, the cows were kept on straw bedding in tiestalls. The cows were offered a TMR and tap water for ad libitum intake. The diet composition was calculated for lactating cows according to the recommendations of the German Society of Nutritional Physiology (GfE, 2001). Feed components and chemical composition of the TMR are shown in Table 1. Cows were milked twice daily at 0400 and 1430 h.

Experimental Procedures

Duodenal cannulas were surgically implanted 4 wk before the start of the bioavailability tests. The PVC duodenal T-cannulas were custom-made by Ludwig Bertram GmbH (Medvet, Laatzten, Germany). The

Table 1. Ingredients and chemical composition (means $\pm$ SE; n = 4) of the diet

Component	Amount
Ingredient (g/kg of DM)	
Grass silage	198.6 $\pm$ 12.56
Corn silage	317.3 $\pm$ 7.80
Barley straw	19.7 $\pm$ 0.00
Hay	19.0 $\pm$ 4.15
Molassed sugar beet pulp ¹	73.5 $\pm$ 6.12
Canola meal extract	11.6 $\pm$ 7.51
Concentrate ²	342.1 $\pm$ 9.82
Supplemented minerals ³	8.5 $\pm$ 0.54
Propylene glycol ⁴	9.7 $\pm$ 1.62
Chemical analysis	
DM (%)	47.71 $\pm$ 1.03
CP (%)	17.23 $\pm$ 0.34
Crude fat (%)	3.03 $\pm$ 0.05
Crude fiber (%)	14.70 $\pm$ 0.16
ME (MJ/kg of DM)	11.60 $\pm$ 0.03
NE _L (MJ/kg of DM)	7.07 $\pm$ 0.03

¹Arp, Thordsen, Rautenberg GmbH & Co KG, Sollerupmühle, Germany: 7.3 MJ of NE_L/kg of DM, 153 g of utilizable protein/kg of DM.

²Concentrate MF 2000 (Vollkraft Mischfutterwerke GmbH, Güstrow, Germany): 33% extracted soy meal, 20% corn, 17% wheat gluten, 8% extracted rapeseed meal, 5% sugar beet pulp, 2% sodium hydrogen carbonate, 1.3% calcium carbonate, 0.2% sodium chloride, 8.0 MJ of NE_L/kg of DM, 204 g of utilizable protein/kg of DM.

³Rinderstolz 9522 lactation (Salvana Tiernahrung GmbH, Sparrieshoop, Germany): 92% crude ash, 20% calcium, 5% phosphorus, 6% magnesium, 8% sodium, vitamins.

⁴Propylene glycol USP (Dr. Pieper Technologie- und Produktentwicklung GmbH, Wuthenow, Germany).

lower disc of the cannulas had an outer diameter of 7.5 cm and the tube protruding from the intestine was 11 cm long with an inner diameter of 2 cm. A hard PVC tube was inserted in the rod, which had a thread to close the cannula to the outside.

After overnight feed withdrawal, the surgical area in the right flank was washed, shaved, degreased, and disinfected with iodine (Vetsept, Albrecht, Aulendorf, Germany). Surgery was performed under local anesthesia, consisting of distal paravertebral anesthesia with 80 mL of 2% procain (Isocain ad us. vet.; procain hydrochloride 20 mg/mL and epinephrine 0.025 mg/mL; Selectavet, Weyarn/Holzolling, Germany), and a cutting-line infiltration anesthesia with another 80 mL of procain. All efforts were made to minimize suffering. A paracostal laparotomy was used to gain entrance to the abdominal cavity. The pylorus, together with the cranial part of the duodenum, was deflected out of the abdomen and the duodenal digesta flow was interrupted with intestinal clamps. The cannulas were implanted in the proximal duodenum approximately 15 cm behind the pylorus. Using a Cushing suture with absorbable thread (polyglycol acid, Catgut GmbH, Markneukirchen, Germany), the duodenum was closely attached to the rod of the cannula and the intestinal clamps were loosened. Abreast the lower part of the incision, about 5 cm cranial, a second 3-cm-long incision was made in the wall of the abdomen. The rod of the cannula was brought to the exterior through this hole and secured by an additional PVC ring with an outer diameter of 9 cm and an interrupted suture that attached the skin to the cannula. After administration of 500 mL of 0.5% iodine solution (diluted Vetsept, Albrecht) into the abdomen, the laparotomy wound was closed.

Pain medication and prophylactic antibiotic treatment were started before beginning of the surgery. For the next 3 d, each cow was treated with analgesics in form of 25 mL of Metapyrin (500 mg of metamizol sodium/mL, Medistar, Ascheberg, Germany) and for 5 d with 60 mL of Trimethosol (40 mg of trimethoprim and 215.8 mg of sulfonamide sodium/mL; Selectavet, Weyarn/Holzolling, Germany). In the periods between flavonol administrations, the cannulas were kept closed by a stopcock with a screw thread.

Cows resumed full feed intake 2.5 d after surgery. Cannulas were cleaned daily during the experimental period. Health status (body temperature, feed intake, milk yield, breathing rate, heart rate, and rumen motility) was monitored daily for 2 wk after surgery.

Before the first bioavailability study, indwelling catheters (Certofix mono, B. Braun Melsungen AG, Melsungen, Germany) were implanted into one jugular vein of the cows when they were between 64 and 83 DIM

for frequent blood sampling. Over the experimental period, the catheters were kept patent for approximately 7 d but were exchanged every other week if necessary. Bioavailability of quercetin was investigated using equimolar amounts of quercetin from 2 different sources: quercetin aglycone (quercetin aglycone dihydrate, **QA**; Carl Roth GmbH, Karlsruhe, Germany) and the quercetin glucorhamnoside (rutin trihydrate, **RU**; Carl Roth GmbH), each applied at 3 dosages, corresponding to 9, 18, and 27 mg of quercetin equivalents (**QE**)/kg of BW. Between 0700 and 0800 h on experimental days, quercetin was administered directly into the duodenal cannula at a rate of 1.8 g/min to avoid disturbance of gut motility. Thirty and 15 min before administration and for 24 h afterward (30, 60, 90, 120, 150, 180, 240, 360, 420, 720, and 1,440 min), blood samples (9 mL) were collected in Li-heparin monovettes (Sarstedt, Nümbrecht, Germany). Basal plasma abundance of quercetin and its metabolites were checked twice per cow after intraduodenal administration of the same volume of isotonic NaCl solution using the same sampling protocol as used for quercetin.

In total, 6 bioavailability tests were conducted per cow in a crossover design, with QA and RU in 3 equimolar doses in alternate order. Between individual bioavailability studies, a washout period of 2 d was allowed to avoid possible carryover effects. Over the whole experimental period, milk yield and feed intake were measured daily.

Sample Processing and Analysis

Dry matter content was determined weekly for grass silage, corn silage, and the TMR. Dry matter, CP, crude fat, and crude fiber in feed samples were determined according to the Weender standard procedure (Naumann and Bassler, 1993). Metabolizable energy and NE_L were calculated according to the German Society of Nutrition Physiology (GfE, 2009).

Blood samples were immediately centrifuged at 2,000 × *g* for 10 min at 4°C. Plasma aliquots were stored at −80°C until analysis of flavonols, glucose, and NEFA concentrations in plasma. Analysis of flavonol content in plasma was done by the Institute of Animal Nutrition and Physiology of the Christian-Albrechts-University (Kiel, Germany). Flavonols were extracted from plasma as described by Egert et al. (2008) and concentrations of quercetin aglycone and its methylated (isorhamnetin, tamarixetin) and dehydroxylated (kaempferol) derivatives were analyzed by HPLC with fluorescence detection as previously described (Ader et al., 2000; Berger et al., 2012). In brief, 980 µL of plasma was acidified with 130 µL of acetic acid (0.583 mol/L),

spiked with 20 μ L of rhamnetin (internal standard, 1 mg/20 mL of methanol; Rotichrom HPLC, Carl Roth GmbH) and treated with a mixture of β -glucuronidase/sulfatase (from *Helix pomatia* Type H-1, with final activities of 7,300 and 130 U/mL for glucuronidase and sulfatase, respectively; Sigma Aldrich Chemie GmbH, Dreieich, Germany) to cleave the ester bonds of glucuronides and sulfates generated through phase II detoxification processes in body tissues. After incubation at 37°C for 60 min, 3 mL of acetone was added and the samples were centrifuged at 4°C for 45 min at $3,700 \times g$. The supernatant was evaporated until dryness (partial vacuum at 45°C, SPD2010 SpeedVac System, Thermo Fischer Scientific GmbH, Dreieich, Germany) and residues were resuspended in 200 μ L of methanol hydroxide. After 15 min in an ultrasonic bath, 77.5 μ L of nanopure H₂O and 22.5 μ L of HCl (10 mol/L) were added. Thirty microliters of the final solution was injected by a cooled (4°C) autosampler (AS-2057 Plus, Jasco, Groß-Umstadt, Germany) onto a C-18 Kromasil 100 column (250 $\times$ 4 mm, particle size 5 μ m, Jasco) guarded by a precolumn (C-18 Inertsil ODS-2, 10 $\times$ 4 mm, particle size 5 μ m, Jasco). The eluent (flow rate: 1 mL/min) was composed of 0.025 mol/L NaH₂PO₄, acetonitrile, and methanol (68:27:5 vol/vol/vol). In a postcolumn reactor, the effluent was mixed with Al(NO₃)₃ [flow rate: 0.4 mL/min, 1 mmol/L in methanol containing 7.5% (vol/vol) acetic acid] for derivatization, and fluorescence of the flavonol-aluminum complex was measured (excitation wavelength 422 nm, emission wavelength 485 nm; FP920, Jasco). Identification of peaks obtained was performed using the retention times of the standards, which were prepared with pure flavonols (Carl Roth GmbH) and treated in the same manner as samples. The detection limit of flavonols was ≤ 10 nmol/L and the recovery rate was $92 \pm 2\%$ (mean $\pm$ SEM). Inter- and intraassay CV for quercetin were 7.2 and 0.5%, respectively.

Glucose and NEFA concentrations in plasma were analyzed by the clinic for cattle of the Tierärztliche Hochschule Hannover using an automatic analyzer (Cobas Mira, Hoffman-LaRoche, Basel, Switzerland) and commercial test kits. Plasma NEFA concentrations were determined using an enzymatic colorimetric assay (No. 434-91795, Wako Chemicals GmbH, Neuss, Germany), and plasma glucose concentrations were measured using the hexokinase method (No. A11A01667, ABX Diagnostics, Montpellier, France).

Calculations and Statistical Analysis

Pharmacokinetic parameters were calculated after correction of each value of the plasma concentration–time curve for the corresponding basal plasma concen-

tration measured 30 min before flavonol administration. Areas under the plasma curve (AUC) were calculated according to the trapezoidal rule between 0 and 1,440 min. Contributions of single flavonol metabolites (quercetin, isorhamnetin, tamarixetin, and kaempferol) to total plasma flavonols were calculated as AUC proportions of the respective AUC of total flavonols (sum of quercetin, isorhamnetin, tamarixetin, and kaempferol). Maximum plasma flavonol concentrations (C_{max}) and the respective time points (T_{max}) were determined directly from the individual plasma concentration–time curves. The apparent terminal plasma elimination half-life (plasma T_{1/2}) was determined using the PK function of Microsoft Excel by linear regression of the terminal portion of the semi-logarithmic plasma concentration–time profiles as described by Graefe et al. (2001).

Data were statistically evaluated using SAS/STAT 9.3 (SAS Institute Inc., Cary, NC). Plasma concentration–time curves of total flavonols and individual plasma flavonols as well as plasma glucose and NEFA concentrations over 24 h were analyzed by repeated-measures ANOVA (PROC MIXED). The model included the fixed factors flavonoid type (QA, RU), dose (0, 9, 18, 27 mg of QE/kg of BW), and the repeated factor time of blood sampling relative to the duodenal administration, as well as the interactions flavonoid type $\times$ dose, flavonoid type $\times$ time, dose $\times$ time, flavonoid type $\times$ dose $\times$ time, and individual cow as random effect. Cow BW, DIM, daily DMI, and milk yield, plasma AUC, T_{max}, C_{max}, and plasma T_{1/2} of single flavonols and total flavonols were also evaluated using the mixed model with the factors flavonoid type and dose and the interaction flavonoid $\times$ dose.

Effects were considered significant at $P \leq 0.05$, and discussed as a trend at $0.05 < P \leq 0.10$. Differences between treatments were identified using the Tukey-Kramer test. Data are presented as least squares means (LSM) $\pm$ standard errors of the mean (SEM).

RESULTS

Bioavailability Studies

Body weight was similar among cows and did not change during the bioavailability studies (Table 2). Twenty-four-hour plasma samples taken during the control studies after administration of physiologic saline showed the presence of quercetin and its metabolites isorhamnetin and kaempferol in plasma at relatively steady amounts, whereas tamarixetin could not be detected. The concentrations of the individual flavonols were within the range of or below the detection limit (Figure 1).

Plasma flavonol concentrations and the pharmacokinetic variables AUC and C_{max} were affected by

flavonoid type, dose, and time of blood sampling ($P < 0.01$; Table 2). Duodenal administration of QA at doses of 9 and 18 mg of QE/kg of BW resulted in increased plasma concentrations of total flavonols after 1.5 h ($P < 0.01$). After administration of the highest dose (27 mg of QE/kg of BW), concentrations of total flavonols were already increased after 1 h ($P < 0.01$; Figure 2). Maximum concentrations ($C_{\max}$) of total flavonols were affected by flavonoid type and increased dose dependently, as was $T_{\max}$, which was between 105 and 125 min (overall LSM 113 ± 6 min) after duodenal QA administration (Table 2). Although interindividual variability of $C_{\max}$ was high at each dosage of QA (396 to 983 nmol/L at the highest dosage administered), $C_{\max}$ was higher with the dose of 27 mg than 9 mg of QE/kg of BW (Table 2). By 3 h after administration of the lowest QA dose, total plasma flavonol concentrations returned to (preadministration) baseline values ($P = 0.28$), whereas for 18 and 27 mg of QE/kg of BW, baseline values were reached 4 h after administration ($P = 0.76$ and 0.47). For individual plasma flavonols, baseline values for kaempferol, quercetin, tamarixetin, and isorhamnetin were reached 150, 180, 180, and 240 min, respectively, after administration of the lowest dose, and were reached 240, 240, 240, and 360 min after administration of the highest dose of QA.

The AUC of plasma total flavonols increased in a dose-dependent manner after administration of QA ($P < 0.01$; Table 2). Based on AUC values after QA administration, quercetin was the main flavonol found in plasma, accounting for 82% of total flavonols measured, independent of the dose applied ($P > 0.9$; Figure 3). The proportion of the methylated metabolite isorhamnetin was an order of magnitude lower than that of quercetin (9%). Tamarixetin and kaempferol accounted for only 4 and 6% of total plasma flavonols, respectively. The relative plasma concentrations of quercetin as well as the proportion of its metabolites (sum of isorhamnetin, tamarixetin, and kaempferol) changed with time ($P < 0.01$). After QA administration, the relative proportion of plasma quercetin (% of total flavonols) was highest at 110 min and decreased steadily until 6 h after duodenal administration (Figure 4).

In contrast to results for QA, duodenal administration of RU did not result in a significant increase of plasma flavonol concentrations over baseline values (Figure 5), and the proportion of the individual flavonols in plasma did not change with time (data not shown).

Effects of Quercetin on Performance and Metabolism

Duodenal administration of QA or RU had no effects on daily DMI (average 21.3 ± 0.2 kg) or milk yield (average 39.5 ± 0.6 kg) on the day of administration

or the next day ($P > 0.9$). Plasma concentrations of glucose and NEFA were not significantly altered within 24 h of the duodenal administration of QA (Figure 6).

DISCUSSION

In bypassing the rumen by the use of duodenal cannulas to avoid microbial degradation, we explored the bioavailability of quercetin from its aglycone form and from rutin in lactating dairy cows. Although quercetin aglycone was absorbed after duodenal administration, the quercetin derived from rutin appeared not to reach the systemic circulation. We anticipated that duodenal administration would result in similar pharmacokinetics for QA and rutin, as has been reported for monogastrics. Indeed, after duodenal administration of comparable doses of QA in cows, the calculated pharmacokinetic variables $C_{\max}$ and $T_{\max}$ for quercetin aglycone were in the same range as found after oral QA administration in pigs (Cermak et al., 2003: 50 mg of QA/kg of W: $C_{\max} = 1,190$ nmol/L, $T_{\max} = 120$ min; Lesser et al., 2004: 10 mg of QA/kg of BW: $C_{\max} = 518$ nmol/L, $T_{\max} = 102$ min). Interestingly, after duodenal administration of QA at 27 mg/kg in cows, the AUC of the plasma concentration–time curve of total flavonols ($95.2 \mu\text{mol/L}$) was only 30% of the AUC in pigs normalized for the same dose (Cermak et al., 2003: 50 mg of QA/kg of BW: AUC = $520 \mu\text{mol/L} \times \text{min}$; Lesser et al., 2004: 10 mg of QA/kg of BW: AUC = $117 \mu\text{mol/L} \times \text{min}$). This implies that quercetin in cows is eliminated or metabolized faster or absorbed less efficiently from the small intestine than in pigs, although the half-life of quercetin calculated in this study (3.3 h) was comparable to that calculated in pigs (3.8 h; Ader et al., 2000). As expected, AUC and $C_{\max}$ were more than 10 times higher after duodenal administration of QA than after intraruminal administration at a similar dose (Berger et al., 2012). Compared with bioavailability parameters after intraruminal administration of rutin, $C_{\max}$, AUC, and $T_{\max}$ values were only slightly higher after duodenal administration of QA if normalized for dose (Berger et al., 2012). Based on these data, rutin might be a rumen-stabilized form of quercetin. Thus, the higher $C_{\max}$ after duodenal administration might suggest that the rumen microbiota not only cleaved the β -glycosidic bond of rutin to yield rutinose and quercetin, but also partially degraded the liberated quercetin to phenolic acid, which might have lowered the systemic availability of quercetin from rutin. The higher AUC after duodenal administration might be due to slower absorption than after intraruminal administration, as suggested by the lower $T_{\max}$ of 42 min after administration into the rumen (Berger et al., 2012).

Table 2. Pharmacokinetic parameters in dairy cows after duodenal administration of 3 doses of quercetin (Q; 9, 18, and 27 mg/kg of BW) as equimolar amounts of the flavonols quercetin aglycone dihydrate and rutin trihydrate¹

Item	Control	Quercetin aglycone			Rutin trihydrate			SEM	<i>P</i> -value		
	0	9	18	27	9	18	27		Flavonol	Dose	Flavonol × Dose
DIM (d)	86.8	87.3	87.3	88.8	87.3	87.3	88.8	3.3	1	>0.9	1
BW (kg)	603.9	602.8	601.5	606.3	602.0	605.8	600.8	8.6	>0.9	>0.9	>0.9
Cmax ² (nmol/L)											
Cmax _{Total}	3.9 ^c	406.9 ^b	573.4 ^{ab}	635.8 ^a	27.1 ^c	18.2 ^c	19.1 ^c	50.2	<0.01	<0.01	<0.01
Cmax _Q	2.9 ^c	350.0 ^b	504.1 ^{ab}	555.5 ^a	21.9 ^c	14.8 ^c	17.3 ^c	44.1	<0.01	<0.01	<0.01
Cmax _K	1.3 ^c	18.2 ^{ac}	19.5 ^{ab}	31.8 ^a	5.5 ^{bc}	2.3 ^c	2.7 ^{bc}	4.2	<0.01	<0.01	<0.01
Cmax _I	0.6 ^b	27.7 ^a	37.0 ^a	34.9 ^a	1.8 ^b	1.8 ^b	1.3 ^b	3.2	<0.01	<0.01	<0.01
Tmax ³ (min)											
Tmax	—	105 ^b	110 ^{ab}	125 ^a	—	—	—	6.1	—	0.058	—
Tmax _Q	—	110	110	125	—	—	—	5.9	—	0.168	—
Tmax _K	—	105 ^b	120 ^{ab}	130 ^a	—	—	—	8.3	—	0.080	—
Tmax _I	—	115	115	135	—	—	—	7.2	—	0.173	—
AUC ⁴ (μmol/L × min)											
AUC _{Total}	2.2 ^c	50.7 ^b	80.0 ^{ab}	95.2 ^a	8.3 ^c	7.9 ^c	6.1 ^c	8.6	<0.01	<0.01	<0.01
AUC _Q	1.3 ^c	40.8 ^b	66.0 ^{ab}	76.1 ^a	5.0 ^c	6.1 ^c	4.0 ^c	6.2	<0.01	<0.01	<0.01
AUC _K	0.6 ^c	3.0 ^{abc}	4.5 ^{ab}	6.3 ^a	2.2 ^{bc}	1.2 ^{bc}	1.8 ^{bc}	0.9	<0.01	<0.01	<0.05
AUC _I	0.3 ^b	5.0 ^b	6.6 ^a	8.7 ^a	1.1 ^b	0.6 ^b	0.5 ^b	1.3	<0.01	<0.01	<0.01
Plasma T _{1/2} ⁵ (h)											
Plasma T _{1/2}	—	2.9	4.8	3.8	—	—	—	1.0	—	0.393	—
Plasma T _{1/2Q}	—	2.8	3.8	3.3	—	—	—	0.8	—	0.661	—

^{a-c}Means in a row without a common letter differ significantly, $P \leq 0.05$.

¹Values are LSM ± pooled SEM; n = 6.

²Cmax = maximum plasma concentration of total flavonols measured less the basal plasma concentration before the duodenal administration; Cmax_Q, Cmax_K, and Cmax_I = maximum plasma concentration of quercetin aglycone, kaempferol, and isorhamnetin, respectively, measured less the basal plasma concentration before the duodenal administration.

³Tmax = time until Cmax was reached; Tmax_Q, Tmax_K, Tmax_I = time until Cmax_Q, Cmax_K, and Cmax_I were reached, respectively.

⁴AUC_{Total} = area under the plasma concentration time–curve (AUC) of total flavonols from time 0 to 1,440 min, calculated according to the trapezoidal rule; AUC_Q, AUC_K, AUC_I = AUC of quercetin aglycone, kaempferol, and isorhamnetin, respectively, from time 0 to 1,440 min, calculated according to the trapezoidal rule.

⁵PlasmaT_{1/2} = terminal plasma half-life of total flavonols; PlasmaT_{1/2Q} = terminal plasma half-life of quercetin aglycone.

Figure 1. Plasma concentration–time curves of flavonols quercetin (●), kaempferol (◇), isorhamnetin (▽), and tamarixetin (■) during control studies (NaCl administration). Values are LSM $\pm$ SEM of 6 cows.

Figure 3. Proportion of single flavonols (quercetin, isorhamnetin, tamarixetin, and kaempferol) in plasma after administration of quercetin aglycone dihydrate (QA), rutin trihydrate (RU), or NaCl (control) derived from total flavonol area under the curve (AUC) values. Proportions were irrespective of dosage of QA and RU, respectively.

Figure 2. Plasma concentration–time curves of total flavonols after administration of 3 doses of quercetin: 9 (▽), 18 (■), and 27 (◇) mg of quercetin equivalent (QE)/kg of BW as quercetin aglycone dihydrate (QA) compared with the control (NaCl solution, ●). Values are LSM $\pm$ SEM of 6 cows for the lower doses (9 and 18 mg of QE/kg of BW) and 5 cows for the highest dose administered (27 mg of QE/kg of BW); * $P < 0.001$ versus preadministration plasma concentrations. Flavonoid type, dose, time, flavonoid type $\times$ dose, flavonoid type $\times$ time, dose $\times$ time, flavonoid type $\times$ dose $\times$ time were significant ($P < 0.001$).

Figure 4. Plasma proportion of quercetin (% of total flavonols)–time curves after administration of quercetin aglycone dihydrate (QA) in 3 doses: 9 (▽), 18 (■), and 27 (◇) mg of quercetin equivalents (QE)/kg of BW. Values are LSM $\pm$ SEM of 6 cows. Error bars were omitted for clarity.

Figure 5. Plasma concentration–time curves of total flavonols after administration of 3 doses of quercetin: 9 (∇), 18 ($\blacksquare$), and 27 ($\diamond$) mg of quercetin equivalents (QE)/kg of BW as rutin trihydrate (RU) compared with the control (NaCl, $\bullet$). Note the difference in scaling of the y-axis compared with quercetin aglycone as presented in Figure 4. Values are LSM $\pm$ SEM of 6 cows. For significant factors, see Figure 2.

The high interindividual variability in C_{max} has also been described in humans and is attributed to a particular polymorphism for intestinal transporters or enzymes (Manach et al., 2005; Egert et al., 2008). Regarding total plasma flavonols in cows, the dominance of quercetin conjugates in plasma and the relatively low concentrations of the methylated metabolites isorhamnetin and tamarixetin after QA administration were also in accordance with findings in humans and pigs (Ader et al., 2000; Egert et al., 2008), whereas more than two-thirds of total flavonols in plasma can be methylated in rats (de Boer et al., 2005).

In contrast to that from QA, the availability of quercetin from RU appears to be negligible in dairy cows when the rumen is bypassed. In monogastric species, absorption of quercetin from rutin compared with quercetin aglycone is delayed because rutinose has to be cleaved by bacterial α -rhamnosidase first in the large intestine. Therefore, absorption is restricted to the large intestine, which explains the lower AUC and C_{max} and higher T_{max} values found in pigs and other monogastric animals (Cermak et al., 2003; Reinboth et al., 2010). One factor contributing to the lack of absorption of quercetin from RU when administered duodenally in dairy cows might be differences between the microbiota of the terminal ileum and large intestine in ruminants and pigs.

Figure 6. Plasma concentration–time curves of (a) glucose, and (b) NEFA after administration of 3 doses of quercetin: 9 (∇), 18 ($\blacksquare$), and 27 ($\diamond$) mg of quercetin equivalents (QE)/kg of BW as quercetin aglycone dihydrate (QA) compared with the control (NaCl, $\bullet$). Values are LSM $\pm$ SEM of 6 cows. Dose and dose $\times$ time were not significant ($P > 0.2$); time was significant ($P < 0.01$).

Although strains of rumen bacteria capable of liberating quercetin aglycone from rutin have been characterized, no data on α -rhamnosidase activity in the hindgut of cattle is available, to the best of our knowledge. Never-

theless, it has been shown that bacterial populations of rumen and large intestine differ greatly in cows (Cheng et al., 1969; Michelland et al., 2009; Frey et al., 2010). However, not only the lack of quercetin aglycone release from RU but also the extensive microbial metabolism of quercetin to phenolic acids could explain the lack of absorption of quercetin after duodenal RU administration. Cermak and Breves (2006) suggested that the microbial activity degrading the flavonol structure could be higher than the activity of α -rhamnosidases in the cecal fluid of slaughtered pigs.

The negligible appearance of quercetin aglycone from rutin in plasma of cows after duodenal application, compared with that in pigs, might also be explained by differences in the retention time of digesta. In cattle, small and large intestinal retention time of digesta accounted only for 24 to 30% of total mean retention time, whereas in the large intestine of pigs, it was 87% of total mean retention time (Mambrini and Peyraud, 1997; Wilfart et al., 2007). Intestinal retention time in cattle has been shown to be lower with higher DMI. In our study, a daily DMI of 21 kg translated to a mean intestinal retention time of approximately 8 to 9 h; this amounts to <3.5 h retention in the large intestine, where degradation of rutin to quercetin is assumed to happen (Pond et al., 1988; Poore et al., 1991; Mambrini and Peyraud, 1997). These values are much lower than those in pigs, where intestinal retention time varies between 28 and 49 h (Pond et al., 1988; Poore et al., 1991; Mambrini and Peyraud, 1997; Wilfart et al., 2007). We assume that low hydrolysis of rutin, due to a short retention time in the large intestine, combined with rapid degradation of the released quercetin aglycone could explain the low bioavailability of quercetin from rutin administered to the duodenum observed in cows compared with pigs.

The occurrence of plasma concentrations of quercetin, isorhamnetin, and kaempferol, albeit low, in cows receiving the NaCl treatment (control) suggests intake of flavonoids with the basal diet. Several studies reported the polyphenolic composition of permanent pastures or various kinds of forages (Fraisie et al., 2007; Besle et al., 2010; Reynaud et al., 2010). Besle et al. (2010) found 0.68 g of quercetin and 0.36 g of kaempferol/kg of DM in ryegrass silage. Ryegrass is widely distributed and native to Europe and therefore might have been part of the grass silage fed in this study. Unfortunately, the flavonol content of the feed was not measured but we estimated a maximal intake of 3 g of quercetin from grass silage in the present study, based on the data of Besle et al. (2010). Because the DMI of cows was not affected by flavonol administration, and flavonol concentrations in plasma during the control studies were very low compared with those achieved by supplement-

ing QA (~20 g/cow at maximal dose), the influence of flavonol intake from the basal diet was assumed to be low.

It has been claimed that feedstuffs rich in phenolic compounds could affect milk yield and quality but the effect of dietary polyphenols has rarely been investigated in ruminants (O'Connell and Fox, 2001). Ewes fed *Lotus corniculatus*, a forage plant rich in proanthocyanidins (27 g of extractable condensed tannin/kg of DM and 8 g of bound condensed tannin/kg of DM), showed increased milk yields, especially in late lactation (Wang et al., 1996). Furthermore, sylimarin (10 g/d), a milk thistle extract consisting mainly of flavonol-lignans, has been shown to increase milk yield and lactation performance if administered for 25 d around calving in dairy cows (Tedesco et al., 2004). However, our finding that a one-time administration did not affect milk yield needs to be verified; in particular, the effects of quercetin after longer term administration need to be studied.

Quercetin supplementation has been shown to affect glucose absorption and metabolism. For example, it reduced blood glucose levels in diabetic rats injected with 10 mg of QA/kg of BW or by supplementing the basal diet with 1 g of QA/kg (Vessal et al., 2003; Shetty et al., 2004). Quercetin has been shown to reduce glucose absorption in vitro at concentrations of 100 μ M in Caco-2 cells by inhibition of sodium-independent glucose uptake (Johnston et al., 2005), and it has been shown that rats treated daily with 10 mg of QA/kg of BW over 10 d showed an increased number of pancreatic islets (Hii and Howell, 1985; Vessal et al., 2003). Further, addition of 10 and 100 μ mol of QA/L enhanced insulin release in isolated pancreatic islets of rats (Hii and Howell, 1985), and quercetin and kaempferol (50 μ M) inhibited glucose-6-phosphatase activity in rat liver (Estrada et al., 2005), which might result in reduced blood glucose levels. However, we did not find significant alterations in plasma glucose or NEFA concentrations after administration of QA. This might be due to several factors. First, effects of quercetin in those studies were evaluated after a long-term dietary supplementation of at least 10 d. Second, in the in vitro studies, comparatively high quercetin concentrations were used, which were not achieved in our in vivo study. Finally, in contrast to that in monogastrics, glucose supply in ruminants depends almost exclusively on gluconeogenesis; hence, impairment of glucose absorption will, if anything, have only minor consequences for plasma glucose concentrations (Bell and Bauman, 1997).

CONCLUSIONS

This study was the first to characterize the bioavailability of quercetin after duodenal administration in

dairy cows. Bioavailability appeared to be within the range observed in monogastric species, and hence a rumen-protected quercetin formulation would be reasonable to avoid ruminal degradation of the flavonol. The data reported here, together with that of the companion paper (Berger et al., 2012), suggest that rutin could be one such rumen-protected form of quercetin. Although we calculated the half-life of total flavonols and quercetin in plasma, this does not necessarily reflect distribution in tissues. Because of the specific nature of this bioavailability study, metabolic data and data on performance warrant verification with longer term applications. In future studies, the diet-derived intake of flavonols should be estimated based on flavonoid analysis in feed.

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