Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf
In cooperation with the Christian Albrechts University Kiel

Oral Bioavailability of Flavonoids and Their Effects on the Metabolic and Antioxidative Status in Neonatal Calves

[Orale Bioverfügbarkeit von Flavonoiden
sowie deren Effekte auf den Stoffwechsel und
den antioxidativen Status beim neugeborenen Kalb]

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Faber est quisque fortunae suae.

(Appius Claudius Caecus)

Content

Chapter 2

Chapter 3

Abbreviations

ASCE	Ascorbic acid equivalent(s)
AUC	Area under the curve
BW	Body weight
BHT	Butylhydroxytoluene
BV	Bioavailability
CA	Catechins
cfu	Colony forming units
C_{max}	Maximal reached plasma concentration
CP	Crude protein
CTRL	Control group, fed no flavonoid
d	Day(s)
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin $F_{2\alpha}$
ELISA	Enzyme-linked immunosorbent assay
FRAP	Ferric reducing ability of plasma
GH	Growth hormone
GLUT	Glucose transporter
GTE	Green tea extract
h	Hour(s)/time of day
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin(s)
IGF	Insulin-like growth factor
IU	International units
LDL	Low-density lipoprotein
LPH	Lactase phlorizin hydrolase
MDAE	Malondialdehyde equivalent
n	Number
NEFA	Non-esterified fatty acids
QA	Quercetin aglycone

QE	Quercetin equivalents
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RU	Quercetin glucorhamnoside rutin
SE	Standard error
SEM	Standard error of the mean
SGLT	Sodium-dependent glucose co-transporter
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidative capacity
TBARS	Thiobarbituric acid reactive substances
T_{max}	time until maximal plasma concentration is reached
U	Unit(s)
wk	week(s)

General Introduction

General Introduction

After birth, calves undergo huge immunological and metabolic changes. Though relatively mature when born they must adapt to various morphological and functional changes after birth (Blum and Hammon, 2000; Hammon et al., 2012). Because of impaired neonatal management like colostrum management, calf losses are highest in the first weeks of life, mainly due to respiratory and digestive diseases. In the United States 2.3 million calf losses were counted in 2010 (USDA, 2011).

Oxidative stress is assumed to play a key role in numerous diseases as almost any disease is somehow related to an increased formation of reactive oxygen species (Halliwell, 1991). In calves, increased levels of markers for oxidative stress were found during the first days of life (Stohrer et al., 2003; Alexandrovich and Antonovna, 2009) and during sickness (Ahmed and Hassan, 2007; Al-Qudah, 2009). Furthermore, the nutrient status highly affects the antioxidant system (Schwerin et al., 2002; Sies et al., 2005) and often is impaired in newborn calves due to the change from intrauterine continuous energy and nutrient supply via placenta to discontinuous oral supply after birth. Thus, improvement of the antioxidant status in newborn calves may contribute to the development of an own defence against environmental threats, which helps to reduce calf losses and to improve health status during the neonatal development.

Because flavonoids as phenolic compounds are claimed to be strong antioxidants and to have countless other health-promoting effects, they are claimed and marketed as health-promoting substances by the feeding industry. Especially since the ban of antibiotic growth promoters in the European Union in 2006, these ‘green’ feed additives gained enormous popularity all over the world. However, any *in vivo* biological effect of a substance requires its bioavailability (BV). In contrast to several monogastric species, where BV of flavonoids is extensively investigated, knowledge on BV of flavonoids in neonatal calves is still lacking. Thus, the aim of this study was to investigate the oral BV of the flavonol quercetin, one of the most abundant flavonoids in nature, fed either as aglycone or as its glycoside rutin in neonatal calves. Studies were carried out on days 2 and 29 of life to show possible differences in absorption, distribution, and metabolism of quercetin during ontogenetic development of newborn calves. Furthermore, oral supplementation with quercetin and catechins for a three-wk period provides

information of flavonoid effects on metabolic and antioxidative status in neonatal calves.

This thesis is structured in 4 major chapters. At first, chapter 1 shows an overview of flavonoids and their BV and introduces major aspects of the antioxidative system and metabolic status in neonatal calves. Chapter 2 deals with the relative oral bioavailability of quercetin fed as quercetin aglycone or as its glucorhamnoside rutin in neonatal calves. Chapter 3 focusses on the effects on the metabolic and antioxidative status in neonatal calves during oral supplementation of quercetin aglycone, rutin, and catechins. Finally chapter 4 presents a general discussion of the results of chapter 2 and 3 in consideration of present knowledge in the literature.

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Chapter 1

Literature Overview

1. Literature Overview

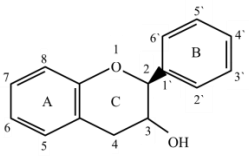
1.1 An introduction in flavonoids

1.1.1 Chemical structure and natural occurrence

Flavonoids are polyphenols, which represent aromatic compounds with one or more hydroxyl groups directly linked to a benzene ring from the common three-ring nucleus. The chemical backbone is based on a C_6–C_3–C_6 carbon framework. Differences on the C-ring divide the flavonoids into six main classes: Flavanols, flavones, isoflavones, flavanones, flavonols, and anthocyanidins (Figure 1.1; Dangles and Dufour, 2006; Manach et al., 2004). Differences between flavonoid classes are predicated by the saturation of the heterocyclic C-ring, which means the presence or absence of a double bond on position 4, the degree of oxidation, and the position of the B-ring (flavones and isoflavones). These variations lead to more than 6,000 different forms of flavonoids known today (Harborne and Williams, 2000). Naturally flavonoids mostly occur as glycoside, which means the C-ring is attached to a sugar moiety. A flavonoid without a sugar moiety is called aglycone. Glycosides yielding glucose instead of other sugar are called glucosides. Only in flavanols the presence of glycosides is rare (Hollman and Arts, 2000). According to Hollman and Arts (2000) aglycones usually do not appear in fresh plants, but after procession. In the class of flavonols there are mainly O-glycosides. The type of sugar bound is predominantly β-glycosidic and the most prevalent sugars next to glucose are arabinose, galactose, and rhamnose.

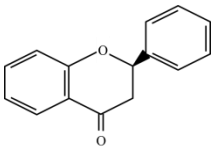
The most abundant flavonoid is the flavonol quercetin (Hollman, 2001; Nijveldt et al., 2001), a pentahydroxyflavon ($C_{15}H_{10}O_7$). Naturally, quercetin is always bound to at least one sugar molecule building quercetin-β-glycoside. The type of sugar differs between plants (Arts et al., 2004). Williams and Harborne (1994) describe about 179 different glycosides of quercetin. The main ones are isoquercetin, rutin, and kaempferol (Figure 1.2).

Flavanol



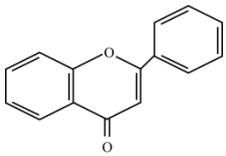
Catechin
Epicatechin
Epigallocatechin gallate

Flavanone



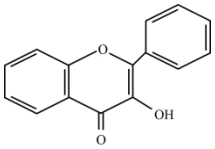
Hesperitin
Naringenin
Eriodictyol

Flavone



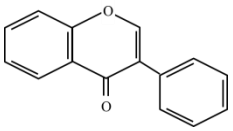
Luteolin
Apigenin

Flavonol



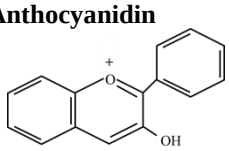
Quercetin,
Kaempferol
Myricetin,
Isorhamnetin

Isoflavone



Genistein
Daidzein
Biochanin A

Anthocyanidin



Pelargonidin
Cyanidin
Delphinidin

Figure 1.1. Basic structure of the six main flavonoid classes with some representatives for each group. In the flavanol structure the numbers and rings are marked.

Modified from Dangles and Dufour (2006); Manach et al. (2004).

Quercetin, a yellow crystalline powder with a molar mass of 302.2 g/mol is hardly water soluble, but soluble in ethanol or dimethyl sulfoxide. The name quercetin is derived from the latin name for oak tree, *Quercus*, where it was found first. Rutin is a glycoside of quercetin bound to rutinose, a disaccharide consisting of rhamnose and glucose. It is also a yellow powder, but less hydrophobic than quercetin. Rutin's molar mass is 610.5 g/mol.

The most abundant flavonols are catechins, which are colorless and slightly water-soluble compounds. They are named after catechu, a juice or an extract of *Mimosa catechu*. Due to its isomer structure, catechins occur as trans- (catechins) and cis-isomers (epicatechins).

In general, flavonoids are ubiquitous plant components in all kinds of higher plants. High amounts of flavonoids can be found especially in onions, broccoli, apples, apricots, tomatoes, and kale as well as in black currants, black and green tea, and red wine (Hertog et al., 1992; Crozier et al., 1997; Hollman and Arts, 2000).

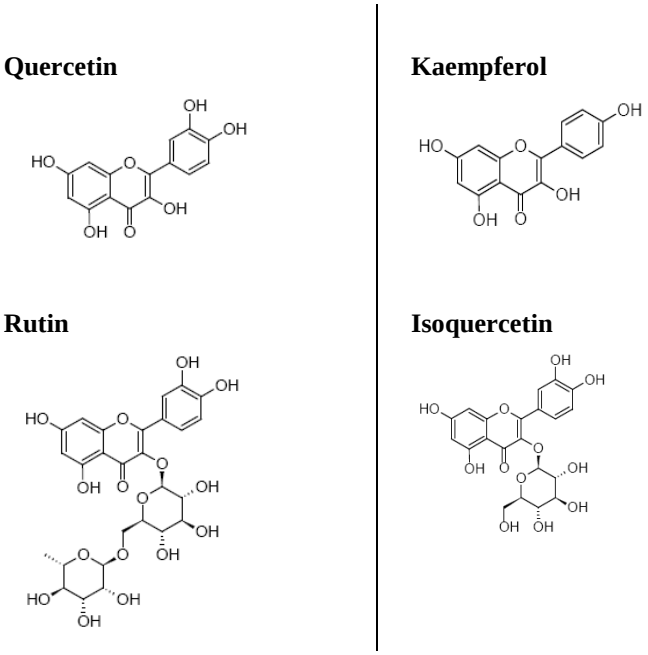


Figure 1.2. Chemical structure of quercetin, kaempferol, and the quercetin glycosides rutin and isoquercetin.

Modified from Passamonti et al. (2009).

An overview of the quercetin, kaempferol, catechin, and epicatechin content in selected food is given in Table 1.1, based on data from the USDA database for the flavonoid content of selected foods, a literature composition based on of 308 different references (Bhagwat et al. 2013) retrieved in December 2013. Further databases on flavonoid content are available, like the Phenol-Explorer (http://phenol-explorer.eu/) or EuroFIR-BASIS (http://ebasis.eurofir.org/), and countless single references giving the flavonoid content in plants.

For chemical determination of the distinct flavonoids, an acceptable separation of the individual flavonoid molecules must be ensured, for example by high-performance-liquid-chromatography (HPLC). However, given values for the flavonoid content in plants can differ enormously depending on methods of analysis, as there is no standardized method for flavonoid analyses. Besides variation of flavonoid content among plants, there are many factors that influence the flavonoid content in plants itself, like seasonal variation, light and climatic conditions, degree of ripeness as well as food preparation and processing (Aherne and O'Brien, 2002). These natural variations in plants, type of plant, and measurement should be kept in mind when comparing flavonoid content among plants.

Table 1.1. Flavonoid content in selected food as is in raw material unless indicated otherwise.

Source	Flavonoid content, mg/kg fresh weight or mg/L			
	Flavonol		Flavanol	
	Quercetin	Kaempferol	Catechin	Epicatechin
Alfalfa, raw seeds	17	0	-	-
Apple	0-115	0-27	0-51	8-192
Apricot	4-29	0-13	3-73	0-83
Buckwheat	51-363	-	-	-
Blackberry	0-119	0-31	0-3129	0-181
Broccoli	0-137	1-213	0	0
Capers	1,493-3,234	2,150-3,083	-	-
Celery	0-35	2	0	0
Cherry, sweet	1-68	0-7	0-149	4-270
Chives	9-104	55-125	-	-
Chocolate, dark	-	-	110-400	520-1,250
Cranberry	73-250	0-6	0-11	30-57
Currant, black	23-122	0-23	7	47
Dock, leaves	862	103	-	-
Elderberry	85-600	2-13	-	-
Grape, black	2-37	0-5	89-108	86-87
Grape seed	-	-	60-2,440	230-2,840
Grape, white/green	1-39	0-3	4-59	1-28
Kale	0-562	5-905	-	-
Milk, chocolate	1	0	1-2	1-5
Onion, red	59-1,917	0-45	-	-
Soybeans[2]	0	12	-	374
Spinach	0-272	0-550	-	-
Tea, black (brewed)	9-48	4-24	4-48	2-87
Tea, green (brewed)	-	-	664-6,880	200-216
wine, red, beverage	0-34	0-13	0-390	0-165

[1]0 = not detectable; - = no data available.
[2]Quercetin and kaempferol content from green soybeans, epicatechin content from mature soybean seeds.

Modified from Bhagwat et al. (2013).

Besle et al. (2010) analyzed phenolic compositions of forages fed to dairy cows and found the greatest phenolic content in grassland pastures with 35.3 g/kg DM. The grassland hay, however, contained 21.6 g/kg DM of phenolic compounds. Ryegrass silage versus ryegrass hay contained 16.8 and 14.5 g/kg DM of phenolic compounds. The lowest phenolic content was found in corn silage containing 3.7 g/kg DM (Besle et al., 2010). Phenolic compounds are also found in trace amounts in bovine milk, wherat their concentration is influenced by cow feeding (Besle et al., 2010; Steinshamn et al., 2008; O'Connell and Fox, 2001).

1.1.2 Biological effects of flavonoids in plants

Flavonoids have multifarious and important functions in plant biochemistry and physiology like protecting deoxyribonucleic acid (DNA) from ultraviolet-induced damage (Kootstra, 1994). Flavonoids further affect photosynthesis by catalysing electron transport processes (Das, 1994). In leaves flavonoid pigments protect the underlying photosynthetic cells by absorbing radiation. Most flavonoids accumulate in the epidermal cell layer of leaves (Winkel-Shirley, 2002) and in the skin of fruits (Manach et al., 2004). The diversity of colors in plants is mainly caused by flavonoids, thus flavonoids play a crucial role in attracting pollinators and seed dispersers (Winkel-Shirley, 2002). Furthermore, flavonoids protect plants against microbial invasion by acting as phytoalexins (Harborne and Williams, 2000), increasing tolerance against abiotic stressors or defend other herbivory, for example by bitter tasting (Gould and Lister, 2006; Harborne and Williams, 2000). The signal transfer between plants and microorganisms is also triggered by flavonoids as seen in the stimulation of the *Rhizobium* bacteria for nitrogen fixation (Gould and Lister, 2006). Flavonoids are stored in plant vacuoles. Cell death causes flavonoids to disperse into different fluid components of the plants like waxes and resins (Das, 1994), which are often considered as enriched with flavonoids.

1.1.3 Biological effects of flavonoids in humans and animals

The effects of flavonoids in humans and animals are seen ambiguous. On the one hand flavonoids are discussed as anti-nutritive factors, whereat on the other hand flavonoids are claimed health and performance promoting. Negative aspects of flavonoids are seen in tannins, where diminishing BV of proteins and minerals or an inhibition of digestive enzymes can occur (Chung et al. 1998). Phytoestrogens are attributed to have adverse health effects in infant development (Zung et al. 2001). However, today's research is mainly focussed on the positive aspects of flavonoids, especially their antioxidative activity and protective capacity against oxidative stress. Flavonoids were found to inhibit oxidation of low-density lipoproteins (LDL) *in vitro* (Harborne and Williams, 2000). Low-density lipoproteins transport lipophilic substances like cholesterol and plasma cholesterol esters. Elevated plasma concentrations of LDL are associated with vascular diseases like atherosclerosis (Aviram and Fuhrman, 2003) and related problems like coronary heart disease and stroke (Denny and Buttriss, 2005). Therefore, a large

body of research deals with the influence of a flavonoid-rich diet on reducing coronary heart and vascular diseases (Hertog et al., 1993; Knekt et al., 1996; Rimm et al., 1996; Yochum et al., 1999; Hirvonen et al., 2001; Mennen et al., 2004). In spite of all these investigations, Loke et al. (2010) postulated the detailed mechanisms of how flavonoids may protect against cardiovascular diseases as still unknown. Besides influencing cardiovascular diseases, flavonoids are involved in cancer control (Lamartiniere et al., 1995; Knekt et al., 1997; Hirvonen et al., 2001). Birt et al. (2001) postulated estrogenic and antiestrogenic activity, antiproliferation, cell cycle arrest, and apoptosis, as well as induction of detoxification enzymes, regulation of host immune function, and other mechanisms of flavonoid actions as responsible for tumor growth restriction. Furthermore, flavonoids are claimed to be involved in modulation of neurodegeneration, especially age-related cognitive and motoric decline, in protection against cerebral ischemia/perfusion injuries or other brain abnormalities (Youdim and Joseph, 2003). Other neurodegenerative disorders in this context are Alzheimer's disease, Parkinson's disease, seizures, trauma, and neuroinflammation (Varadarajan et al., 2001; Schroeter and Spencer, 2003; Miller et al., 2009). In addition, flavonoid treatment may alleviate infection by human immunodeficiency virus, because flavonoids are capable to inhibit several critical enzymes involved in life cycle of human immunodeficiency virus (Cos et al., 2008).

Effects of flavonoids and their metabolites on the metabolism in organisms are manifold. Especially those on carbohydrate metabolism are well studied and an impact on glucose homeostasis was found in several studies. Hanhineva et al. (2010) reviewed the impact of dietary polyphenols on the carbohydrate metabolism and summarized the inhibition of carbohydrate digestion and glucose absorption in the intestine, the stimulation of insulin secretion from pancreatic β-cells, the modulation of glucose release from the liver, the activation of insulin receptors and glucose uptake in insulin-sensitive tissues as well as the modulation of intracellular signalling pathways and gene expression as possible mechanisms of how flavonoids influence carbohydrate metabolism. More in detail, flavonoids are capable to inhibit α-amylase and α-glucosidase activity (Tadera et al., 2006), inhibit the sodium-dependent glucose co-transporter (SGLT) 1 (Kobayashi et al., 2000) or glucose transporters itself (Johnston et al., 2005). In rat insulinoma pancreatic cells polyphenols like quercetin, apigenin, and luteolin inhibited cytotoxicity and attenuated the decrease of glucose-stimulated insulin

secretion (Kim et al., 2007). However, insulin secretion is affected by a wide range of different plant compounds and extracts. Glucose uptake can be stimulated by flavonoids in both, insulin-sensitive and non-insulin sensitive tissue, mostly dose-dependent (Hanhineva et al., 2010). Glucose transporter (GLUT) 1, widely distributed in fetal tissues, and GLUT 4, expressed by muscle, are supposed to mainly trigger flavonoid mediated glucose uptake (Hanhineva et al., 2010).

However, the influence of polyphenols on liver function to maintain glucose homeostasis may also play a key role in neonates. Endogenous glucose production as important pathway to ensure glucose homeostasis in the neonate (Girard et al., 1992) originates from glycogenolysis and gluconeogenesis, both taking place in the liver. An improved hepatic glucose and lipid metabolism was indicated in diabetic rats feeding a polyphenol-rich diet (Roghani and Baluchnejadmojarad, 2010; Bose et al., 2008; Wolfram et al., 2006). In non-diabetic rats, quercetin stimulated glycogenolysis and inhibited glycolysis and gluconeogenesis (Gasparin et al., 2003a; b) *ex vivo* by infusing the liver with 50 to 300 µmol/L quercetin. However, these concentrations are not achievable with standard diets for *in vivo* experiments (Gasparin et al., 2003a, b). Furthermore, Kobayashi et al. (2010) and Seiva et al. (2012) showed positive effects of a flavonoid-rich diet on lipid metabolism in rats fed a high-fat diet as plasma concentrations of triglycerides, non-esterified fatty acids (NEFA), insulin, and leptin as well as lipogenesis were reduced.

1.2 Bioavailability of flavonoids

1.2.1 General aspects of bioavailability with focus on ruminants

In general, BV can be defined as the rate and extent to which the active ingredient of a substance is absorbed in the blood compartment or becomes available at the site of action. The European Agency for the Evaluation of Medicinal Products (2000) defines BV as the extent and rate to which a substance or its active moiety is delivered from a pharmaceutical form and becomes available in circulation. However, the effect of a substance is usually determined by its concentration at the place of action.

All postabsorptive biological effects of given substances depend on their BV. Parameters determining BV are liberation, absorption, distribution, metabolism, and excretion, abbreviated with their first letters as LADME-system. Liberation, the first

step for an effect of a drug or an active compound, means drug release or drug delivery and can be seen as a complex process. The drug must disaggregate in smaller particles, dissolve in the present fluid, and disperse, which is more difficult for water insoluble substances like flavonoids. In the next step, the absorption, the active compound must enter the blood or lymph vessels when given orally. Absorption of orally given ingredients is determined by solution rate as well as gut and stomach content/filling (Frey, 2007). Bioavailability was shown to be influenced by technological (galenical) factors of the substance, the form of application, local factors like diarrhea or contact with other substances, and first-pass effects. These first-pass effects occur when an active compound is metabolized very fast and thus inactivated or transformed before entering the systemic circulation, for example in the gut mucosa or liver. After absorption into the bloodstream, the active compound is distributed by passive diffusion or by convective transport from the blood capillaries into neighboring tissues. The metabolism of an active compound means its biotransformation, mainly increasing its hydrophilic properties (Pfeiffer et al., 1995). The main organ for biotransformation is the liver, but it also takes place in the mucosa cells of the gastrointestinal tract, lung, and kidney. Most enzymes involved in this process are located in the endoplasmic reticulum (Frey, 2007). Biotransformation is two-phased. Phase-I-reaction is a transformation where functional groups are built into non-polar molecules mostly by oxidation, reduction or hydrolysis. Absorption to water soluble molecules in phase-II-reaction enables excretion. The most important ways of excretion or elimination are renal, biliary, and intestinal.

To determine BV, the concentration of an active ingredient is measured over time in blood samples collected from the systemic circulation, and as plasma concentration-time curves are specified only by absorption, distribution, and elimination. To calculate BV, area under the curve (AUC), maximal plasma concentration (C_{max}), and time until the maximal plasma concentration is reached (T_{max}) are used as pharmacokinetic parameters (see Figure 1.3).

Bioavailability is either relative or absolute. For determination of the absolute oral BV of a substance, the AUC after oral intake is compared to the AUC after intravenous injection. In contrast to this, relative oral BV compares two different drugs, containing the same active principle after oral application. As time interval of sampling mainly determines accuracy, sampling frequency should be as high as possible.

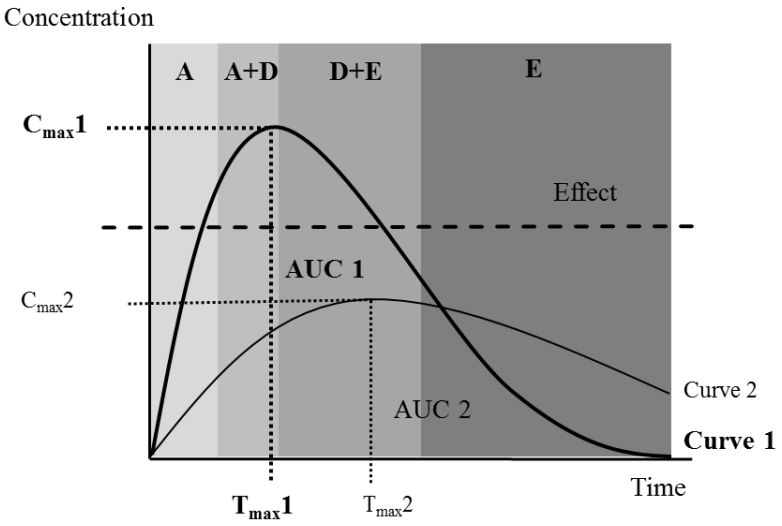


Figure 1.3. Illustration of main pharmacokinetic parameters at 2 different plasma concentration-time curves after a single oral dose of an active compound to an organism. Bold descriptions are for curve 1.

A = absorption; D = distribution; E = elimination; AUC = area under the curve; C_{max} = maximal plasma concentration; T_{max} = time until maximal plasma concentration is reached.

Modified from Pfeiffer et al. (1995).

1.2.2 Absorption and distribution of flavonoids in the organism

Flavonoids can be absorbed in different ways after oral administration from the gastrointestinal tract. Quercetin fed as aglycone without attached sugar can be detected within 30 minutes after oral administration in plasma of volunteers, whereas quercetin fed as rutinoside with attached sugar (rutin) showed delayed peak levels in plasma (Hollman et al., 1997b; Olthof et al., 2000; Cermak et al., 2003). Thus, the main place of absorption of quercetin monoglycosides is the small intestine. More complex quercetin glycosides like rutin are mainly absorbed in the colon or the distal small intestine, also colonized with microorganisms (Hollman et al., 1997b; Manach et al., 1997). Therefore, the sugar moiety and the location of microbial activity in the gastrointestinal tract account for different absorption sites (Hollman et al., 1999; Day et al., 1998). Some flavonoid aglyca can already be absorbed in the stomach, but not their glycosides, as shown in studies in rats administered quercetin (Crespy et al., 2002) or daidzein and genistein (Piskula et al., 1999). However, most flavonoid glycosides resist acid hydrolysis in the stomach thus reaching the duodenum undegraded.

To pass the gastrointestinal epithelium is the first crucial step for systemic availability in an organism. The enterocytes are polarized cells with numerous membrane proteins

that enable the transport of dietary compounds from the lumen into the blood stream via channels or active transporters (Figure 1.4). Lipophilic and small nonpolar substances can pass the plasma membrane via diffusion following a concentration gradient. As flavonoids are polar and thus hydrophilic substances the passive diffusion of flavonoids, however, barely occurs in the gastrointestinal tract (Day et al., 2000; Manach et al., 2004; Crespy et al., 2003). Only for the aglyca a passive absorption is discussed (Day et al., 2000; Cermak et al., 2003; Wolffram, 2010). Acidic conditions in the stomach inhibit hydrolysis (Day et al., 2000) and pancreatic enzymes are also not able to hydrolyze β-glycosidic bounds (Arts et al., 2004). On the contrary Walle et al. (2005) found hydrolysis of dietary flavonoids already in the oral cavity by both, bacteria and shed epithelial cells, but with a large interindividual variability between volunteers. Furthermore, Kahle et al. (2011) found degradation of polyphenols varying after incubation with human saliva. Different glucosidases like lactase phlorizin hydrolase (LPH) are known to hydrolyze flavonoids (Day et al., 2000; Sesink et al., 2002; Németh et al., 2003). Lactase phlorizin hydrolase is an extracellular enzyme from the brush border membrane of intestinal mammalian cells and was shown to be important for intestinal absorption of quercetin glycosides (Figure 1.4; Sesink et al., 2002). Extracellular glucosidases cleave the sugar from the flavonoid glucosides and the aglycone can be absorbed. Hydrolysis by lactase phlorizin hydrolase might be reduced in rats and sheep due to their higher microbial population in the stomach than in humans (Day et al., 2000). In human small intestine and liver an intracellular, cytosolic β-glucosidase was described that hydrolyze various flavonoid glycosides (Day et al., 1998). Further enzymes hydrolyzing flavonoids in the gut are glucocerebrosidase (Day et al., 1998) and other cytosolic β-glucosidases (McMahon, 1997). More complex glycosides, like rhamnosides, are hydrolyzed by enzymes of microorganism in the distal small intestine or in the colon (Crespy et al., 1999; Scholz and Williamson, 2007). Besides that, active transport systems are prevalently used for flavonoid transport into the cell (Figure 1.4). Several studies suggest that quercetin glucosides could be absorbed in the small intestine via SGLT 1 (Gee et al., 1998; Walgren et al., 2000a; Wolffram et al., 2002). Also GLUT, like GLUT 2 (Chen et al., 2007), are discussed to transport quercetin glucosides across the cell membranes. Since the membrane transport of xenobiotics, chemical compounds like antibiotics that are neither produced in an organism nor are part of the regular diet, is a key factor in medical treatment and

flavonoids may interact with the cellular in- and efflux systems like the multidrug-resistance-associated protein, a plethora of studies with the interaction of flavonoids and the membrane transport of drugs or their interactions exists (Cermak and Wolffram, 2006). Among plasma membrane transporters, the focus is on adenosine triphosphate-binding cassette transporters (di Pietro et al., 2002; Brand et al., 2006; Morris and Zhang, 2006), monocarboxylate transporters, and organic anion and bilirubin transporters (Figure 1.4; Passamonti et al., 2009).

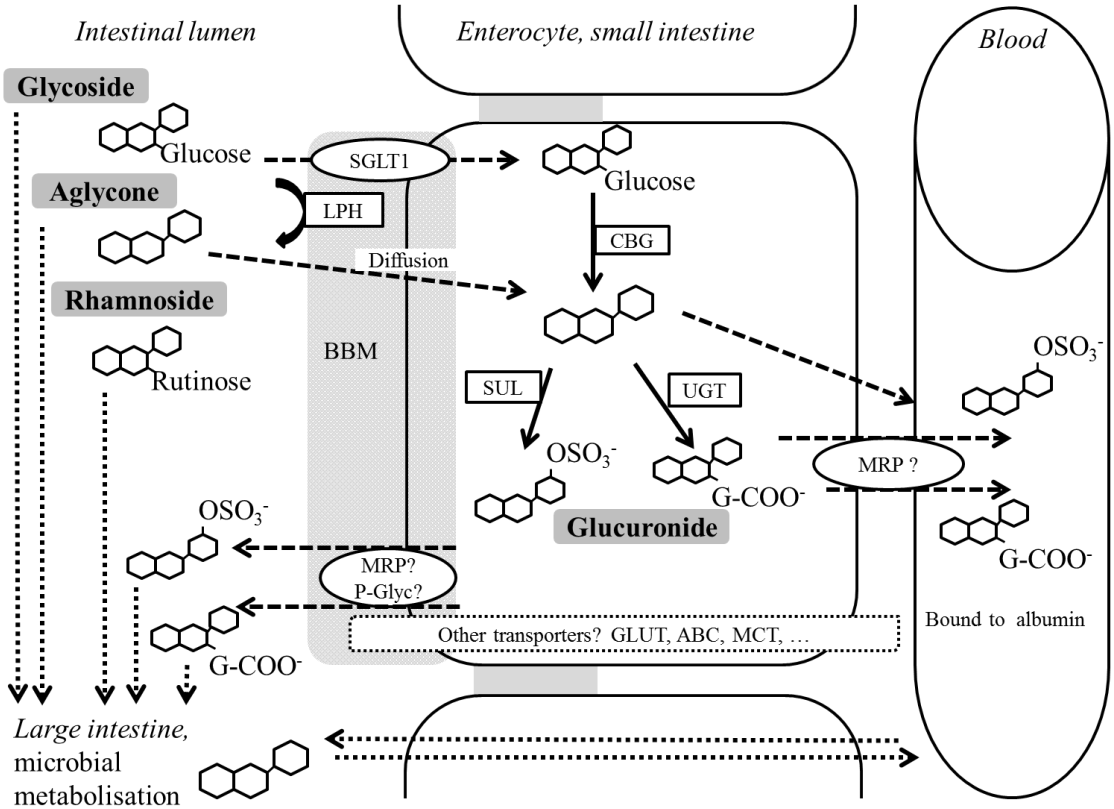


Figure 1.4. Simplified model of intestinal absorption and metabolism of the flavonol quercetin in the organism.

Solid arrow = enzymatic reactions; dotted or dashed arrow = active or passive transport; SGLT1 = sodium-dependent glucose co-transporter 1; LPH = lactase phlorizin hydrolase; CBG = cytosolic β-glucosidase; UGT = uridine-5´-diphospate glucuronosyl-transferase; MRP = multidrug resistance-associated protein; SUL = sulfotransferase; GLUT = glucose transporter; ABC = ATP-binding cassette transporter; MCT = monocarboxylate transporter; P-Glyc = *P*-glycoprotein.

Modified from Murota and Terao (2003); Petri et al. (2003); Cermak and Wolffram (2006).

However, the exact way of action of these membrane proteins on flavonoid transport *in vivo* is not given yet. Once absorbed into circulation, flavonoids are distributed in the body with more than 98 % of quercetin bound to proteins (Gugler et al., 1975; Boulton

et al., 1998; Janisch et al., 2004), mainly albumin as the most prevalent protein in plasma. Further, quercetin is also bound to glycoproteins and LDL (Boulton et al., 1998). Depending on their conjugation, the affinity of quercetin to bind on serum albumin differs (Janisch et al., 2004) contributing to the huge variation in BV (Dufour and Dangles, 2005). However, as it is known that binding of the polyphenol to proteins alter their biological activity, the biological effect of this binding needs still to be evaluated (Manach et al., 2004).

The pattern of metabolites in tissues may be different from the one in the blood stream due to its cellular uptake or intracellular metabolism (Manach et al., 2004). Furthermore, Manach et al. (2004) consider that plasma concentrations do not necessarily correlate with the concentration in tissue. Thus, plasma concentration of flavonoids may not be an accurate biomarker of exposure to body tissue.

1.2.3 Metabolism and excretion of flavonoids

First-pass effect during intestinal absorption is crucial for availability of orally administered flavonoids as described in chapter 1.2.1. Hydrolyzation of some flavonoids already occurs by passing the enterocytes via the membrane-bound lactase phlorizin hydrolase. Other flavonoids are hydrolyzed in the enterocyte by cytosolic β-glucosidase before entering the systemic circulation (Day et al., 2000, 1998; Sesink et al., 2002) and being transported to the liver. Flavonoids reaching the colon are hydrolyzed by microorganisms before absorption. In human intestine the presence of the bacteria species E*ubacterium*, C*lostridium*, and B*acterioides* indicated metabolism of flavonoids. Various simple aromatic acids are produced in this process and the released aglycone is often degraded by colonic microorganisms (Manach et al., 2004). These aglycones are further degraded by splitting the heterocyclic ring, thus flavonols mainly produce hydroxyphenylacetic acids. Different flavonoid classes lead to different acids, like flavones and flavanones are mainly metabolized to hydroxyphenylpropionic acids. All breakdown products are further metabolized to derivatives of benzoic acid; microbial metabolites are absorbed in the colon and conjugated with glycine, glucuronic acid or sulfate (Manach et al., 2004). Due to the different composition of the colonic microflora in different species the variations in microbial metabolism of flavonoids are enormous.

The main site of flavonol metabolism besides the gastrointestinal tract is the liver (Hollman and Katan, 1997; Aherne and O'Brien, 2002). Flavonoids are transported to the liver via the portal vein. During absorption in the intestine and later in the liver flavonoids are conjugated by phase-II-reactions, mainly glucuronidation, methylation, and sulfation. Phase-I-reactions like oxidation by enzymes of the cytochrome P450 system are of minor importance in flavonoid metabolism (Otake et al., 2002). The reactions of phase-II mainly are detoxificating processes in the body like metabolism of xenobiotics (Manach et al., 2004). When methylated, a methyl group of quercetin is transferred, catalyzed by the catechol-*O*-methyl transferase (Manach et al., 2004), which is found in many tissues, with highest activity in liver and kidneys (Piskula and Terao, 1998). Glucuronidation occurs in the liver, but also to a great extent in the intestine (Spencer et al., 1999). This process is catalyzed by the Uridine diphosphate-glucuronosyltransferase, which is located in the endoplasmatic reticulum. Here, glucuronic acid from Uridine diphosphate-glucuronic acid is transferred to the polyphenol. Sulfation also mainly occurs in the liver (Piskula and Terao, 1998). The transfer of a sulfate moiety from 3´-phosphoadenosine-5´-phosphosulfate to the hydroxyl group of polyphenols is catalyzed by sulfotransferases. According to the kind of the substance and the ingested dose, the importance of glucuronidation, methylation, and sulfation varies (Manach et al., 2004). A higher ingested dose causes a shift from sulfation to glucuronidation (Koster et al., 1981). However, the pattern of conjugation is further influenced by species, sex or food deprivation (Piskula, 2000).

Flavonoids formed in the gut mucosa only partially enter circulation and a significant amount will be secreted back into the gut lumen (Crespy et al., 1999). This so called apical efflux out of the enterocytes is probably triggered by adenosine triphosphate-binding cassette transporter, like the multidrug resistance associated protein 2 (Walle et al., 1999; Walgren et al., 2000b), the breast cancer resistance protein 1 (Sesink et al., 2005) or the P-glycoprotein (Ofer et al., 2005).

However, flavonoids often undergo an intensive enterohepatic circulation. Due to their biliary excretion back into the duodenum, flavonoids may be further metabolized by intestinal microorganisms or reabsorbed from the intestine (Hackett, 1986). This recycling may enhance availability of flavonoids in the body (Manach et al., 2004) or cause a second plasma peak (Setchell et al., 2001; Manach et al., 2003). According to the site of absorption, flavonoids may undergo different metabolic fates, thus the liver

might be more important for flavonoids absorbed in the small intestine than for the ones absorbed in the colon (Heim et al., 2002).

Flavonoids and their metabolites usually are excreted by bile or urine. The smaller conjugates prefer urinary excretion, whereas the larger and extensively conjugated metabolites prefer the biliary one (Manach et al., 2004). Flavonoid glucuronides and sulphates are readily excreted with the urine due to their polarity and water solubility (Hackett, 1986). However, though various flavonoids are excreted by urine (Aherne and O'Brien, 2002; Scalbert and Williamson, 2000; Manach et al., 2004) this route seems to be of minor importance (Hollman et al., 1995). Due to partially long elimination half-life flavonoids may be accumulated in blood plasma because of repeated absorption (Aherne and O'Brien, 2002).

1.2.4 Factors influencing bioavailability of flavonoids

Bioavailability of flavonoids may be influenced at all states of the described LADME-system on both, pre- as well as post-absorptive by species differences and individual factors like state of development and nutrition (Heaney, 2001), enzyme activities or secretion, other regulatory mechanisms, and interindividual differences (Egert et al., 2008). Plasma peak concentrations of epigallocatechin gallate increased 3.5-fold when catechins were given to fasted compared to non-fasted volunteers (Chow et al., 2005). Further, mucosal mass, intestinal transit time, rate of gastric emptying as well as the up- and down-regulation of absorption by physiological control because of habituation influenced flavonoid BV (Heaney, 2001). Summarized by Scholz and Williamson (2007), the most important criterion influencing BV is the attached sugar, followed by the food matrix, the added flavonoids, the given dose and the adaption to it, and the inhibition of flavonoids by chemical changes after absorption or processing in the gastrointestinal tract or due to interactions with other feed components.

The effects of the food matrix, the physical form in which flavonoids are administered, and solubility on BV seem to be more complex. Quercetin is slightly soluble in water, but addition of organic solvents such as lipids, emulsifiers (Azuma et al., 2002) or propylene glycol (Shimoi et al., 1998) may enhance solubility. The benefit of alcohol on BV of flavonoids is discussed ambiguous as some found appreciable improvements (Azuma et al., 2002; Dragoni et al., 2006), others not (Donovan et al., 1999; Goldberg et al., 2003). However, Azuma et al. (2002) only enhanced BV of flavonoids using

alcohol concentrations of more than 30 % in the diet. Overall, composition of the meal and the source of flavonoids have the main impact on BV. Quercetin from onions had a higher BV than quercetin from apples or pure rutin (Hollman et al., 1997b). Food preparation and processing like cooking also affect BV since it can cause a reduction in flavonoid content or cause chemical transformation (Manach et al., 2004). Furthermore, flavonoid absorption is affected by the fat content in the diet (Lesser et al., 2004). Interestingly, interactions of polyphenols with milk proteins partly inhibited positive aspects of flavonoids or led to differences in absorption or reduced AUC values of flavonoid plasma concentrations (Serafini et al., 2003, Reddy et al., 2005; Egert et al., 2013). Nevertheless, other studies in humans showed no effect of milk on BV of flavonoids (van Het Hof et al., 1998; Hollman et al., 2001).

1.2.5 Studies on bioavailability of quercetin and catechins

In cattle, the companion studies of Berger et al. (2012) and Gohlke et al. (2013), where quercetin aglycone and rutin were given intraruminal (10 and 50 mg/kg body weight (BW); Berger et al., 2012) and intraduodenal (9, 18 or 27 mg/kg BW; Gohlke et al., 2013) to dairy cows, showed maximal plasma concentrations of flavonols ranging from about 20 to 1,000 nmol/L at 30 min to 2 h after administration. Interestingly, rutin was much better available than quercetin aglycone after intraruminal (Berger et al., 2012), than after intraduodenal administration (Gohlke et al., 2013). Manach et al. (2005) reported a range from 0 to 4 µmol/L plasma concentration of total flavonols after intake of 50 mg aglycone equivalents in humans, whereas an older study of Gugler et al. (1975) found no effect of oral administration on quercetin plasma concentration in humans, and therefore suggested oral administration of quercetin as ineffective. However, more recent studies revealed detectable plasma concentrations of quercetin after oral flavonol administration as shown in Table 1.2. In this overview only studies were listed in which pure quercetin substances were administered and plasma concentrations were measured. Though lots of BV studies are conducted with food containing high contents of flavonoids like apples, wine or onions, interactions with other food components or matrix effects cannot be excluded. Therefore, it is more reliable using pure substances for comparing BV studies.

Studies on the BV of catechins with pure substances are rare, as mostly tea extracts were used. According to Manach et al. (1999), in rats the catechin metabolites (catechin

and methylcatechin) reached similar plasma concentrations after oral supplementation of catechin than the quercetin metabolites quercetin, isorhamnetin, and tamarixetin after quercetin administration in comparable doses. However, metabolites of catechin showed a faster decline in plasma concentration than those of quercetin, possibly due to lesser binding to plasma albumin or higher urinary excretion (Manach et al., 1999). Hollman et al. (1997a), too, postulated a good absorption of catechin and its microbial degradation products after oral administration of radioactively labelled (+)-catechin to rodents, monkeys, and humans in different studies. Nevertheless, BV of catechins widely depends on species, on the administered compound, and on the chemically structure of the distinct catechin isomers, such as epicatechin, epigallocatechin, and epigallocatechin gallate. In rats fed 200 mg/kg BW of a green tea extract (GTE), Chen et al. (1997) found a systemic availability of 31.2 % for epicatechin and of 14 % for epigallocatechin, but only of 0.1 % for epigallocatechin gallate, whereas in dogs after oral and intravenous administration of 25 mg/kg BW of epigallocatechin gallate absolute BV was about 20 % (Swezey et al., 2003). However, systemic available epicatechin, epigallocatechin, and epigallocatechin gallate was very low after oral administration of 20 mg/kg BW of green tea to humans (Lee et al., 2002) or after oral administration of 500 mg/kg BW and intravenous administration of 50 mg/kg BW catechin fractions containing 5 % epicatechin, 13 % epicatechin gallate, and 50 % epigallocatechin gallate to rats (Zhu et al., 2000). The consumption of a green tea beverage by humans resulted in maximal plasma concentrations of total catechins of 461.8 ± 27.4 nmol/L at 90 min after drinking (Egert et al., 2013). Major catechins in this study detected in plasma samples after consumption of various test drinks were epigallocatechin gallate (179.9 nmol/L), epicatechin gallate (147.3 nmol/L), epigallocatechin (86.5 nmol/L), epicatechin (48.2 nmol/L), and small amounts of catechin and gallocatechin (Egert et al., 2013).

Table 1.2. Overview of the bioavailability of quercetin from different natural forms.

Reference	Subject, application	Flavonoid, dose	Converted data		
			AUC, nmol/L × h	T_{max}, min	C_{max}, nmol/L
Berger et al. (2012)	Cow, intraruminal	Q, 50 mg/kg BW	182	30	90
		Q, 10 mg/kg BW	-	30	~20
		R, 50 mg/kg BW	1,399	42	1,000
		R, 10 mg/kg BW	-	30	~80
Gohlke et al. (2013)	Cow, intraduodenal	Q, 9 mg/kg BW	845	105	407
		Q, 18 mg/kg BW	1,333	110	573
		Q, 27 mg/kg BW	1,587	125	636
		R, 9 mg/kg BW	138	-	27
		R, 18 mg/kg BW	132	-	18
		R, 27 mg/kg BW	102	-	19
Cermak et al. (2003)	Pig, oral	Q, 50 mg/kg BW	8,677	20	1,190
		R, 50 mg/kg BW	1,997	210	357
Lesser et al. (2004)	Pig, oral	Q, 10 mg/kg BW	1,950	102	518
Ader et al. (2000)	Pig, oral	Q, 50 mg/kg BW	-	240	852
		Q, 500 mg/kg BW	-	150	4,113
Erlund et al. (2000)	Human, oral	Q, 8 mg	1,744	114	137
		Q, 20 mg	2,918	162	219
		Q, 50 mg	3,765	294	285
		R, 8 mg	624	1,410	38
		R, 20 mg	1,042	444	78
		R, 50 mg	1,666	450	147
Hollman et al. (1997b)	Human, oral	R, 200 mg	1,610	558	147

Table 1.2. Continuation.

Reference	Subject, application	Flavonoid, dose	Converted data[1]		
			AUC, nmol/L × h	T_{max}, min	C_{max}, nmol/L
Hollman et al. (1999)	Human, oral	Spiraeosid, ~150 mg	18,800	30	3,500
		Rutosidum, ~200 mg	3,700	360	180
Olthof et al. (2000)	Human, oral	Isoquercitrin, ~150 mg	19,100	37	5,000
		Spiraeosid, ~150 mg	17,500	27	4,500
Egert et al. (2008)	Human, oral	Q, 50 mg	1,268	120	189
		Q, 100 mg	3,000	180	295
		Q, 150 g	5,097	360	431
Manach et al. (1997)*	Rat, oral	Q, 0.2 % of diet	-	720	55,000
		R, 0.4 % of diet	-	1,440	35,000
Manach et al. (1999)	Rat, oral	Q, 0.25 % of diet	-	1,440	52,000
Reinboth et al. (2010)	Dog, oral	Q, 10 mg/kg BW	2,915	1) 72 2) 234	1) 233.6 2) 229.2
		R, 10 mg/kg BW	2,667	1) 42 2) 666	1) 17 2) 113.7
		Isoquercitrin, 10 mg/kg BW	6,837	1) 48 2) 246	1) 667.4 2) 888.3
Wein and Wolffram (2013)	Horse, oral	Q, 20 mg/kg BW	2,010	60	750

[1]AUC, T_{max} and C_{max} are converted all into same units for comparison of data.
R = quercetin as glucorhamnoside rutin; Q = quercetin as aglycone (without attached sugar); AUC = area under the plasma-concentration time curve; C_{max} = maximal reached plasma concentration of total flavonols measured in each study; T_{max} = time till C_{max} is reached; BW = body weight; - = no values given; * = data evaluated from figure; 1), 2) = first and second peak.

1.3 Metabolic and health status in neonatal calves

1.3.1 Metabolic status in calves at birth and postnatal changes

Although calves are precocial animals and thus relatively mature when born, they still must adapt to various morphological and functional changes after birth (Blum and Hammon, 2000). During gestation, energy supply is continuously via placenta with glucose as main energy substrate. With birth this changes to a discontinuously oral supply by colostrum and milk intake with lactose and fat as main energy source (Hammon et al., 2013). Prenatal, glucocorticoids and catecholamines as well as thyroid hormones in the fetal blood lead to an increased gluconeogenic activity and hepatic glycogen storage ensuring coverage of the energy demand of the newborn calf (Hammon et al., 2012). However, calves are often hypoglycemic after birth, as they need to adapt to the usage of other fuels than glucose, taken up with liquid feeding. Thus, endogenous glucose production is an important pathway to ensure glucose homeostasis in the calf and increases with maturation (Steinhoff-Wagner et al., 2011). Important regulators for endogenous glucose production are hormones like insulin, glucagon, catecholamines, growth hormone, and glucocorticoids. The peptide hormone insulin is built in the pancreatic β-cells. Plasma concentrations are reduced at birth and increase after first feed intake indicating that especially glucose intake stimulates insulin secretion (Hadorn et al., 1997; Hammon and Blum 1998; Rauprich et al., 2000). The peptide hormone glucagon is built in the pancreatic α-cells and acts as antagonist to insulin. Plasma glucagon concentrations also increase after first feed intake in calves (Hammon et al., 2012). Endogenous glucose production is in addition stimulated by catecholamines, glucocorticoids, and probably growth hormone (GH). Around birth, all these hormones showed elevated plasma concentrations in calves and catecholamines and glucocorticoids are known for their promoting effects on metabolic maturation in the neonate (Hammon et al., 2012, 2013). Plasma concentrations of cortisol are high at birth and decrease thereafter (Hadorn et al., 1997; Hammon et al., 2012; Schiessler et al., 2002). In addition, cortisol, next to other regulators, is a major player for initiating birth (Liggins, 1994). Furthermore, thyroid hormones play an important role for endogenous glucose production during late gestation and after birth (Fowden et al., 2001). Plasma concentrations of thyroid hormones are high at birth and decrease

thereafter in mature calves, but not in preterm born calves (Steinhoff-Wagner et al., 2011).

In neonatal calves colostrum feeding affects the GH – insulin-like growth factor (IGF) system, which most likely is a response to energy supply and improved glucose status in calves after colostrum feeding (Hammon et al., 2012). Amount, time point, and frequency of feeding influence plasma IGF-1 concentrations (Hadorn et al., 1997; Nussbaum et al., 2002), which are lower during the first wk of life in restricted (Hadorn et al., 1997) or formula fed calves (Rauprich et al., 2000) indicating the huge importance of early and sufficient colostrum feeding to newborn calves for their development and maturation.

1.3.2 Health status in calves and development of the immune system

Immune defence of newborn calves depends on uptake of immunoglobulins (Ig) with colostrum as there is no placental transfer of Ig and endogenous production of Ig is negligible (Chase et al., 2008). The ingestion of sufficient colostrum is thus essential for immunological protection during the first 2 to 4 weeks of life until calves develop an adequate own active immune system (Chase et al., 2008; Figure 1.5). With colostrum Ig are transferred from the cow to the newborn calf and activate and regulate the innate immune response (Chase et al., 2008).

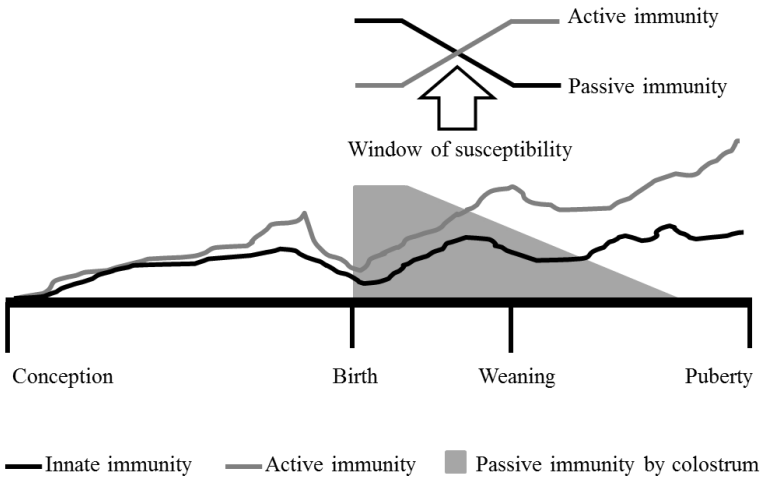


Figure 1.5. Illustration of the development of immunity and immune response of calves.
Modified from Chase et al. (2008).

Apart from nutrients and Ig colostrum contains minerals, vitamins, and also non-nutrient components like growth factors, cytokines, hormones, and further bioactive

substances (Blum, 2006). Most of these non-nutrient factors and bioactive compounds are derived from blood of dams, but some are produced in the mammary gland (Blum, 2006). The average Ig concentrations in bovine colostrum varies greatly, depending mainly on breed, age of dam, nutrition in the preparturient period, season or amount of produced colostrum (Godden, 2008). As colostrogenesis ends abruptly at parturition the greatest concentration of the beneficial compounds is in first colostrum and in further milkings concentrations are decreasing because of dilution effects (Godden, 2008). The small intestine as the site of digestion and absorption of Ig, mainly IgG, is permeable for up to 12 to 48 h after birth (Stott et al., 1979; Baintner, 2007). Feeding the calf leads to a faster closure of the gut permeability for great Ig, independent from the amount fed (Stott et al., 1979). Small intestinal epithelium includes vacuoles that allow intracellular digestion at birth, but soon after colostrum ingestion vacuoles disappear (Blum, 2005). Therefore, colostrum influences the gastrointestinal tract as seen among others in changes in microbial population, epithelial cell proliferation, protein synthesis, and digestion (Blum, 2006). Furthermore, colostrum also exerts long-lasting systemic effects on metabolism and endocrine traits by effecting systemic growth, activity, and behavior (Blum, 2006).

Endogenous Ig production and thus active immunity in calves is delayed (Chase, 2008). With passive immunity decreasing and active immunity still being insufficient susceptibility to diseases is highest in the first wk of life (Figure 1.5), and notable calf losses occur with pneumonia and diarrhea being the main reasons for it (USDA, 2011). Pneumonia in pre-weaned calves is a multi-factorial disease caused by a number of viruses and some calf- and environmental related risk factors (Lorenz et al., 2011). Diarrhea is one of the most serious problems in calf rearing worldwide often leading to other diseases like pneumonia in enfeebled calves. Furthermore, those calves show reduced growth. So calves suffering from diseases in newborn age may show long-lasting impaired performance.

1.3.3 Oxidative stress and antioxidative capacity in neonatal calves

Oxygen is essential for all aerobic living organisms. It is part of several chemical compounds like hydrogen peroxide, hypochlorus acid, peroxinitrite, singlet oxygen, and ozone. Importantly, oxygen has the potential to destroy cell integrity when building up reactive oxygen species (ROS). These ROS can be divided in free radicals and non-

radicals. In general, a free radical is an atom or molecule with one or more unpaired electrons (Halliwell, 1991), marked by the radical dot (·). The most reactive ones are hydroxyl radical, superoxide anion, nitric oxide, and peroxyl radical. All these ROS are naturally produced in the body during energy production and unspecific immune defense, mainly to neutralize bacteria and viruses, and thus protecting the body against pathogens. External influences like ionizing radiation, toxins, drugs, and chemicals or environmental pollutants can considerably increase the generation of ROS (Sies, 1991) resulting in cell toxic conditions.

The organism has a pool of reducing systems to neutralize these free radicals, called antioxidants. These are substances that, when present at low concentrations compared with those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate (Halliwell, 1991). Antioxidants are divided into enzymatic antioxidants, like superoxide-dismutase, catalase or glutathion-peroxidase, and non-enzymatic antioxidants, including albumin, carotenoids, bilirubin, uric acid, glutathion, vitamins A, C, and E as well as flavonoids. Every aerobe living system is aligned to have a balance between these oxidants and antioxidants, but due to inadequate antioxidant defense or external influences, an imbalance between the generation of prooxidants and the activity of the antioxidant defenses in favor for the prooxidants can occur and is called oxidative stress (Sies, 1985, 1991). Oxidative stress is related to numerous diseases, the so called free radical diseases. According to Halliwell (1991) for most human diseases oxidative stress might be secondary to the primary disease process. Next to maturation of the organism (Robles et al., 2001; Saugstad, 2005; Mutinati et al., 2014) nutrient status and nutrition in general plays a major role in the antioxidant system (Schwerin et al., 2002; Sies et al., 2005). However, neonates have a greater susceptibility to oxidative stress than adults or juvenile subjects (Saugstad, 2005; Mutinati et al., 2014). Main reason therefor is the change from the hypoxic intrauterine environment to extrauterine conditions after birth, and the start of regular breathing (Robles et al., 2001; Albera and Kankofer, 2011). Especially premature neonates are prone to oxidative stress (Robles et al., 2001).

There are several approaches to measure antioxidative capacity, oxidative stress or free radicals. Alam et al. (2013) reviewed 29 different *in vivo* and *in vitro* methods for evaluating antioxidant activity and oxidative stress. In general, common methods range from quantification of (free) radicals, quantification of markers for oxidative stress, like

DNA damage or lipid peroxidation, up to the quantification of markers for the antioxidant system, like enzymes. Figure 1.6 gives an overview of the most common methods for evaluation of oxidative stress and antioxidant capacity. In general, the antioxidative status should not only be evaluated by a single test model since there is no absolute method (Alam et al., 2013).

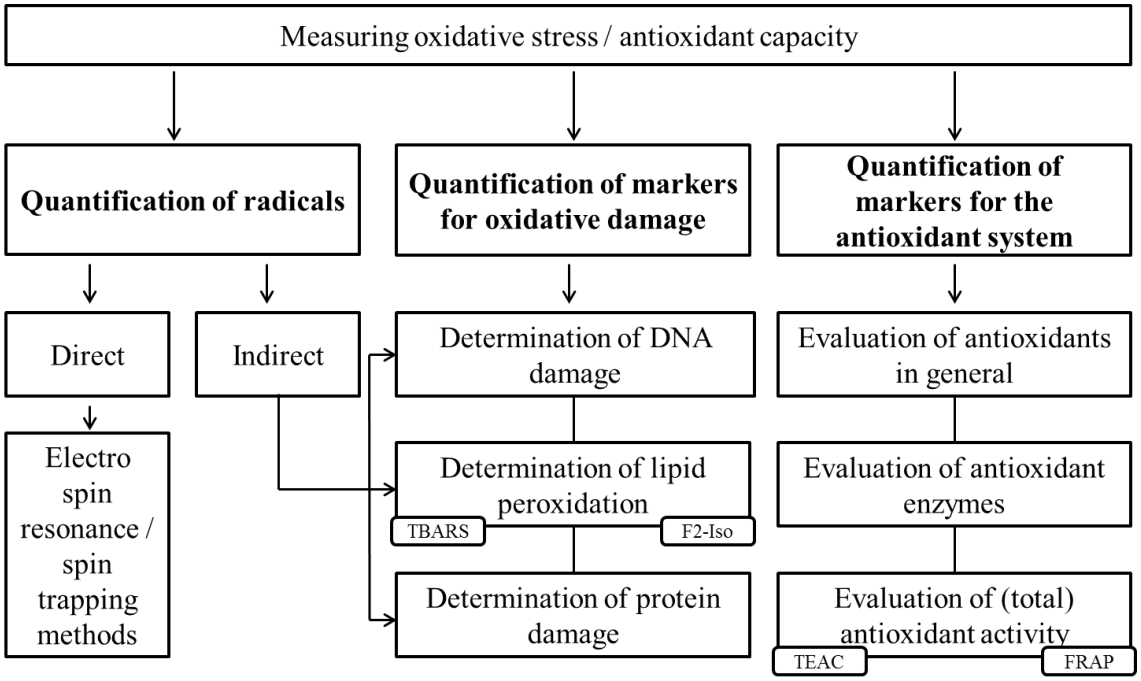


Figure 1.6. Simplified classification of methods used for determining oxidative stress and antioxidant capacity.

TBARS = thiobarbituric acid reactive substances; F2-Iso = F2-isoprostanes; TEAC = trolox equivalent antioxidative capacity; FRAP = ferric reducing ability of plasma.

Modified from Kohen and Nyska (2002).

There are plenty of studies describing the antioxidative status or oxidative stress in calves, but only a few studies can be compared directly due to different methods of analyzing and experimental set-up. Main studies in calves deal with selected parameters of oxidative stress or the antioxidant capacity. Stohrer et al. (2003) investigated the antioxidant status of cows and calves via trolox equivalent antioxidative capacity (TEAC) and found impairment during the first wk of life and thus assumed that ischemia/reperfusion injury during birth may affect antioxidative status of neonatal calves. Alexandrovich and Antonovna (2009) confirmed an elevated level of oxidative stress during the first d of life up to d 45 of age due to an increased level of

thiobarbituric acid active products and glutathione when calves were less than 15 d old, and reduced values when calves were 50 to 60 d old. The authors assume that the transition period in feeding of calves was the reason for that. Also transportation of calves leads to an increase of markers for oxidative stress: Wernicki et al. (2006) found significantly elevated thiobarbituric acid reactive substances (TBARS) plasma concentration in calves that were transported for 2 h. The season did not significantly change TBARS levels in calves, but glutathione peroxidase and superoxide dismutase were higher in calves born in summer than in calves born in fall (Chigerwe et al. 2013). However, in this study no correlation between TBARS and the age of calves was found, but there was an obvious correlation between increased oxidative stress or a reduced antioxidative capacity and health status of calves. These findings confirmed a study by Ahmed and Hassan (2007), who investigated several parameters of calves infected with *Eimeria* species. Malondialdehyde and nitric oxide as markers for oxidative stress were elevated in infected calves. Catalase, superoxide-dismutase, ascorbic acid, glutathione, and the total antioxidant capacity were significantly reduced in infected calves compared to control calves. Bronchopneumonia as a major calf disease similarly showed elevated markers of oxidative stress in calves (Al-Qudah, 2009). Also the way of delivery seems to influence the antioxidative status since higher malondialdehyde and glutathione concentrations in blood showed an exposure to higher oxidative stress in calves delivered by caesarian section (Erisir et al., 2013). Retskii et al. (2010) 'corrected' the antioxidant status of newborn calves to form higher colostral immunity due to treatment with glucose, ascorbic acid solution, and unithiol and thus reduced the incidence and severity of neonatal diseases.

1.3.4 Effects of flavonoid administration on development and health status in neonatal calves

Countless flavonoid-rich feed additives are marketed claiming improvements in animal health and productivity in cattle nutrition. These additives are used in calves' milk replacer and in feed for heifers, lactating cows, and fattening cattle (http://sanogold.eu/news/2008/flavonoide.php; http://www.pulte.de/Antioxidantien_Botanische_Wirkstoffe&lang=en; http://www.schaumann.de/cps/rde/SID-18 120AAB B3-717B45/schaumann-de/hs.xsl/4736.html; http://www.provimi.com.gr/media/grape_pp_concentrate_-_eng.pdf; http://www. gainfeeds.com/ruminant/ goldgrain-calf-nut).

In general, lots of feed additives are supposed to reduce or replace antibiotics, thus terms like phytobiotics, phototherapeutic or nutraceutical are commonly used. Since flavonoids are present in all kinds of higher plants, young ruminants may have evolutionary depended on flavonoids (Yaghoubi et al., 2008). However, results of studies on feeding flavonoids to young calves are ambiguous. As mentioned, the metabolism of calves mainly is influenced by nutrition; hence Yaghoubi et al. (2008) postulated an improved growth of calves by feeding flavonoids extracted from propolis. Enhancing growth in ruminants by flavonoid feeding is discussed due to possible effects on microbial population in the rumen (Aerts et al., 1999; Greathead, 2003) and especially in the developing reticulorumen (Yaghoubi et al., 2008; Costa Jr. et al., 2012). According to Aerts et al. (1999) the degradation of dietary protein to ammonia by microorganisms in the rumen of sheep is slowed down by proanthocyanidins, which are polymere forms of catechins, and thus increasing the absorption of amino acids in the small intestine. Durmic and Blache (2012) also reviewed beneficial effects of secondary plant compounds on rumen fermentation, focused on the protection of dietary proteins combined with a reduced microbial proteolysis and release into the duodenum. However, mainly tannins are building complexes with proteins (Aerts et al., 1999). In general, secondary plant metabolites may exert prebiotic effects due to stimulation of certain bacteria in the rumen (Greathead, 2003) and affect the immune system as hypothesized by Yaghoubi et al. (2008) though the authors found an ambiguous blood IgG concentrations after feeding flavonoids to calves. Metwally et al. (2013) reported a numeric increase in blood IgM concentrations after feeding propolis extract to buffalo calves, considering a non-specific immunostimulating effect. Also Oliveira et al. (2010) postulated an improved immune response of calves when feeding a flavonoid-rich pomegranate extract due to an enhanced total IgG response after ovalbumin vaccination and increased synthesis of interferon-γ and interleukin-4. On the other hand, neutrophil phagocytic and killing activities of neutrophils against *Escherichia coli* did not differ when feeding pomegranate extract to young calves (Oliveira et al., 2010). Besides that, some flavonoids have hormone-like structures and activities and therefore are called phyto-oestrogens (Greathead, 2003) probably exerting anabolic effects in calves. Such effects were seen in other species like mice (Sokolova et al., 1978). Furthermore, it is noteworthy that quercetin is a major component of several plants used as antidiarrheal remedies (Middleton et al., 2000). Di Carlo et al. (1994) showed quercetin feeding

ameliorating diarrhea in mice by 92 % in a dose-related manner as did Gálvez et al. (1995) in quercitrin (3-rhamnosylquercetin)-treated rats, and Nielsen (2008) in piglets. Rao et al. (1997) pointed out a possible antidiarrheal effect of the flavonoid ternatin. However, propolis or pomegranate extract feeding did not ameliorate diarrhea in calves (Yaghoubi et al., 2008; Oliveira et al., 2010), but feeding a GTE decreased frequency and degree of non-pathogenic diarrhea remarkably (Ishihara et al., 2001).

1.4 Conclusive remarks and need for further research

Green feed additives like flavonoids have a considerably role in human and animal nutrition. Numerous feed supplements containing flavonoids are marketed in the dairy industry to improve health, growth, and thus productivity.

These antioxidative, anti-inflammatory and antimicrobial secondary plant metabolites may especially be used in the neonatal period when calves are just developing their immune system. However, little is known about the systemic BV of flavonoids and their effects *in vivo*, especially in calves. Thus, the aim of this study is to investigate the BV of the most abundant flavonoids quercetin and catechin in newborn calves and to examine their effects on selected aspects of the antioxidative status and metabolism. Therefore, dairy calves were fed quercetin as aglycone, its glucorhamnoside rutin, and catechins for the first 3 wk of life. Parameters of the antioxidative status, oxidative stress, and metabolism were determined and studies on BV of quercetin aglycone and rutin were performed on d 2 and 29 of life, representing a different ontogenetic status in calves.

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Chapter 2

Bioavailability of the flavonol quercetin in neonatal calves after oral administration of quercetin aglycone or rutin

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2. Bioavailability of the flavonol quercetin in neonatal calves after oral administration of quercetin aglycone or rutin

Abstract

Polyphenols, such as flavonoids, are secondary plant metabolites with potentially health-promoting properties. In newborn calves flavonoids may improve health status, but little is known about the systemically availability of flavonoids in calves to exert biological effects. The aim of this study was to investigate the oral bioavailability of the flavonol quercetin, applied either as quercetin aglycone (QA) or as its glucorhamnoside rutin (RU), in newborn dairy calves. Twenty-one male newborn German Holstein calves were fed equal amounts of colostrum and milk replacer according to body weight. On d 2 and 29 of life, 9 mg of quercetin equivalents/kg of body weight, either fed as QA or as RU, or no quercetin (control group) were fed together with the morning meal. Blood samples were taken before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 12, 24, and 48 h after feed intake. Quercetin and quercetin metabolites with an intact flavonol structure (isorhamnetin, tamarixetin, and kaempferol) were analyzed in blood plasma after treatment with glucuronidase/sulfatase by HPLC with fluorescence detection. Maximum individual plasma concentration was depicted from the concentration-time-curve on d 2 and 29, respectively. Additional blood samples were taken to measure basal plasma concentrations of total protein, albumin, urea, and lactate as well as pre- and postprandial plasma concentrations of glucose, non-esterified fatty acids, insulin, and cortisol. Plasma concentrations of quercetin and its metabolites were significantly higher on d 2 than on d 29 of life, and administration of QA resulted in higher plasma concentrations of quercetin and its metabolites than RU. The relative bioavailability of total flavonols (sum of quercetin and its metabolites isorhamnetin, tamarixetin, and kaempferol) from RU was 72.5 % on d 2 and 49.6 % on d 29 when compared with QA (100 %). Calves fed QA reached maximum plasma concentrations of total flavonols much earlier than did RU-fed calves. Plasma metabolites and hormones were barely affected by QA and RU feeding in this experiment. Taken together, orally administrated QA resulted in a greater bioavailability of quercetin than RU on d 2 and 29, respectively, and differed markedly between calves aged 2 and 29 d.

Key Words: bioavailability, calf, flavonoid, quercetin, rutin

2.1 Introduction

Flavonoids are secondary plant metabolites occurring ubiquitously in all higher plants (Manach et al., 2004; Besle et al., 2010). They are known for their health-promoting properties (e.g. antioxidative and anti-inflammatory; Middleton et al., 2000; Nijveldt et al., 2001; Williams et al., 2004). Quercetin is one of the most abundant flavonoids and is present in high concentrations in onions, apples, and kale (Hertog et al., 1992; Nijveldt et al., 2001), and in low concentrations also in milk (Besle et al., 2010; Bhagwat et al., 2013). In addition to their health-promoting properties, quercetin and its metabolites modulate the expression and activity of several metabolic key enzymes, and therefore might be involved in regulation of lipid and carbohydrate metabolism (Middleton et al., 2000; Gasparin et al., 2003; Kobayashi et al., 2010).

Newborn calves undergo tremendous immunological and metabolic changes after birth to adapt for extra-uterine life (Blum, 2006; Chase et al., 2008; Hammon et al., 2012) and colostrum management is one of the most important factors to support neonatal health and development (Godden, 2008; Hammon et al., 2012). Nevertheless, morbidity and mortality rates are still high during first weeks of life and calves often suffer from diarrhea and respiratory disease (McGuirk, 2008; Mee, 2008; Uetake, 2013) as well as from high levels of oxidative stress (Inanami et al., 1999; Gaál et al., 2006). Frequent problems on farms are the lack of high-quality colostrum availability and the insufficient colostrum supply (Quickley and Drewry, 1998; Godden, 2008). Colostrum and mature milk contain antioxidant agents to protect neonatal calves from oxidative stress (Lindmark-Månsson and Åkesson, 2000; Besle et al., 2010). Poor quality of colostrum is reflected by low concentrations of antioxidative substances. Thus, supplementing colostrum and milk at beginning of lactation with the natural antioxidant agent quercetin may improve neonatal oxidative status. However, there is no information about the bioavailability (BV) of orally applied quercetin with colostrum and milk in newborn calves, although feeding industry already offers flavonoid-supplemented feed for dairy calves.

Studies on BV of quercetin from quercetin aglycone (QA) or its glucorhamnoside rutin (RU) in different monogastric species, such as rats (Manach et al., 1997), pigs (Ader et al., 2000; Cermak et al., 2003; Lesser et al., 2004), dogs (Reinboth et al., 2010), and humans (Erlund et al., 2000; Egert et al., 2008), showed marked differences to ruminant

species (e.g. cows with intraruminal QA and RU application; Berger et al., 2012). These differences may be due to differnces in gastrointestinal anatomy and physiology (Arts et al., 2004; Berger et al., 2012; Gohlke et al., 2013). Conversely, in newborn calves the forestomach system is just developing, thus newborn calves are functionally monogastrics (Drackley et al., 2008). The aim of the present study was to investigate the relative BV of quercetin after oral administration of QA or RU in calves during first month of life. We hypothesized that BV in calves depends on the form of application (QA and RU) and changes with age due to ontogenetic development and maturation of the gastrointestinal tract during first month of life. We further tested the hypothesis that quercetin application might affect metabolic and endocrine traits, especially concerning glucose metabolism in neonatal calves, because findings in literature pointed to impair carbohydrate digestion and glucose absorption after flavonoid intake (Cermak et al., 2004; Tadera et al., 2006).

2.2 Materials and Methods

2.2.1 Animals and feeding

The experimental procedures were carried out according to the animal care guidelines and were approved by the relevant authorities of the State Mecklenburg-West Pomerania, Germany (LVL M-V/TSD/7221.3-2.1-019/10). Twenty-one male German Holstein calves were examined on d 2 and 29 of life. All calves were spontaneously born from multiparous cows on neighboring farms and transported directly after birth to the experimental barn. Calves were kept in single boxes with straw bedding and had free access to water. Calves were fed twice daily (0700 and 1500 h) with a nipple bottle or nipple bucket. On the first 3 d of life calves received pooled colostrum obtained from milkings 1, 3, and 5 (d 1, 2, and 3 after parturition, respectively; Table 2.1) at amounts of 8 % of BW on d 1 and 10 % of BW on d 2 and 3 (Steinhoff-Wagner et al., 2011). From d 4 until d 29, calves received milk replacer (150 g/L; SalvaLac MiraPro 45, Salvana Tiernahrung GmbH, Klein-Offenseth Sparrieshoop, Germany) at 12 % of BW/d (Table 2.1). To ensure uptake of equal amounts of feed, refused amounts of colostrum or milk were tube-fed to calves. Milk intake was adapted to BW data once a week.

Colostrum or milk replacer was supplemented with chicken egg-derived immunoglobulins (Globigen Life Start 25 %, EW Nutrition GmbH, Visbek, Germany) composed of 75 % dextrose and 25 % whole egg powder (10.75 % CP, 10.50 % crude fat, 0.10 % crude fiber, and 2.50 % ash) with high antibody titer against *Eschericha coli* type K 99, *Salmonella* Tymphimurium and *Salmonella* Dublin, bovine rotavirus type G6 and G10, bovine coronavirus, *Cryptosporidium parvum*, and *Clostridium perfringens* serotype C. Immunoglobulins were added from d 2 till d 6. Respective amounts of immunoglobulins fed twice daily were 40, 32, 24, 16, and 8 g/d.

From d 4 on calves had free access to pelleted concentrate (Kälber Start 18/3 pell., Vollkraft Mischfutterwerke GmbH, Karstädt, Germany; Table 2.1) and hay. Concentrate intake was measured daily after morning milk feeding. To avoid iron deficiency, calves received 600 mg iron dextran subcutaneously (Ursoferran, Serumwerk Bernburg, Germany) on their first day of life. Navel disinfection was performed with 10 % iodine solution (vet sept Lösung, Albrecht GmbH, Aulendorf, Germany) immediately after birth. Health status of calves was determined daily by measuring rectal temperature, heart rate, and respiratory rate, by evaluation of behavioral abnormalities, nasal discharge, respiratory sounds, fecal consistence, and by navel inspection.

2.2.2 Treatment and blood sampling

Calves were randomly assigned to 1 of 3 feeding groups (n = 7 per group) receiving either no flavonoids (control group; **CTRL**), 9 mg of **QA**/kg of BW (quercetin aglycone dihydrate, Carl Roth GmbH, Karlsruhe, Germany), or 18 mg of **RU**/kg of BW (rutin trihydrate, Carl Roth GmbH), each resulting in a dose of 9 mg of quercetin equivalents (QE)/kg of BW on d 2 and d 29 of life. Calves received the whole dose of QA or RU during morning feeding, applying the QE suspension with a 10-ml syringe directly into the mouth.

Table 2.1. Composition and amounts of colostrum, milk replacer, and concentrate fed to calves.

Item	d 1 colostrum[1] milking 1	d 2 colostrum[1] milking 3	d 3 colostrum[1] milking 5	d 4 to 30[2] Milk replacer[3]	d 4 to 30[2] Concentrate[4]
DM, g/kg	206.9	154.7	139.3	110.0	880.0
CP, g/kg DM	597.4	390.4	333.1	201.8	180.0
Crude fat, g/kg DM	169.2	240.5	274.9	190.9	43.0
Ash, g/kg DM	48.3	55.6	56.0	73.6	74.0
Lactose, g/kg DM	136.3	260.5	308.7	514.6	-

[1]Colostrum was derived from multiparous cows and separately pooled from milkings 1, 3, and 5 after parturition, respectively.
[2]Hay was offered ad libitum except for days of bioavailability studies.
[3]Milk replacer (SalvaLac MiraPro 45, Salvana Tiernahrung GmbH, Klein-Offenseth Sparrieshoop, Germany), was composed of 45 % spray skimmed milk powder, 35 % sweet whey powder, 18 % plant oil (palm, coconut and soy oil), 1.7 % Lys, 0.8 % Ca, 0.75 % P, and added with 50,000 IU of vitamin A, 4,000 IU of vitamin D_3, 200 mg of vitamin E (α-tocopherol acetate), 9 mg Cu (glycerine-copper chelate), citric acid, sorbic acid, butylhydroxytoluene (BHT), and 1.2 x 10^9 cfu *Enterococcus faecium* E1706.
[4]Concentrate (Kälber Start 18/3, Vollkraft, Mischfutterwerke GmbH, Karstädt, Germany) was composed of wheat gluten, wheat bran, grain mash, sugar beet pulp, rapeseed meal, oat, rye, molasses, linseed, oat bran, soybean meal extract, calcium carbonate, sodium chloride, 0.6 % phosphorous, 0.2% sodium, 10,800 IU of vitamin A, 1,215 IU of vitamin D_3, 30 mg of vitamin E, 0.7 mg of I, 0.4 mg of Co, 54 mg of Mg, 81 mg of Zn, and 0.4 mg of Se per kg of DM.

The day before the study started, a catheter (Certofix Mono 340, Braun Melsungen AG, Melsungen, Germany) was inserted into the calves' right jugular vein and blood samples were taken before (time point 0), and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, and 48 h after feeding using S-Monovette tubes (Sarstedt AG & Co., Nümbrecht, Germany) containing lithium heparin (16 IU/mL blood) for analysis of plasma flavonols. Additional blood samples, except on 1.5, 2.5, and 48 h, were taken for measurement of plasma concentrations of total protein, albumin, glucose, NEFA, urea, and lactate using tubes containing sodium fluoride and K_3EDTA (1.0 mg/mL fluoride and 1.2 mg/mL EDTA). Blood sampled into tubes containing dipotassium EDTA (1.8 mg/mL) was used for determination of insulin and cortisol plasma concentrations. Catheters were flushed with 10 mL sodium chloride solution (0.9 % sodium chloride, Braun Melsungen AG) after each blood sampling. Blood was immediately put on ice, centrifuged for 20 min at 1,500 × *g* at 4° C, and plasma was stored at -80° C for flavonol analysis, and at -20° C for analyses of metabolites and hormones.

2.2.3 Analytical methods

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; Gohlke et al., 2013). Briefly, plasma samples were thawed in a 40° C water bath. Then 980 µL of plasma was acidified with 130 µL of acetic acid (0.583 mol/L), spiked with 20 µL of methanolic rhamnetin solution (internal standard, 1 mg/20 mL in methanol; Rotichrom HPLC, Carl Roth GmbH) and treated with a mixture of β-glucuronidase and 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. After incubation at 37° C for 1 h, 3 mL of acetone was added and the samples were centrifuged at 3,700 × *g* for 45 min at 4° C). The supernatant was evaporated until dryness. Residues were resolved in 200 µL methanol, and after 15 min in an ultrasonic bath 77.5 µL of nanopure water and 22.5 µL of hydrochloric acid (10 mol/L) were added. For flavonol analyses by HPLC, 30 µL of the final solution was injected by a cooled (4° C) autosampler (AS-2057 Plus, Jasco Deutschland GmbH, Groß-Umstadt, Germany) onto

a C-18 Kromasil 100 column (250 × 4 mm, particle size = 5 µm, Jasco Deutschland GmbH) guarded by a pre-column (C-18 Inertsil ODS-2, 10 × 4 mm, particle size = 5 µm, Jasco Deutschland GmbH). The eluent (flow rate: 1 mL/min) was composed of 0.025 mol/L sodium dihydrogen phosphate (pH 2.4), acetonitrile, and methanol (68:27:5 vol/vol/vol). In a postcolumn reactor, the effluent was mixed with aluminum nitrate [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 using a fluorescence detector (excitation wavelength = 422 nm, emission wavelength = 485 nm; FP920, Jasco Deutschland GmbH). Identification of peaks obtained was performed using the retention times of the standards, which were prepared with pure flavonols and treated in the same way as samples. The detection limit of flavonols was ≤10 nmol/L and the recovery rate was 92 ± 2 % (mean ± SE). Inter- and intraassay variances for quercetin were 7.2 and 0.5 %. The sum of plasma concentrations of quercetin and its metabolites in plasma is referred to as total flavonols.

Plasma metabolites were analyzed spectrophotometrically (ABX Penta 400; Horiba ABX SAS, Montpellier Cedex, France) by the Clinic for Cattle (University of Veterinary Medicine, Foundation, Hanover, Germany), using the respective kits: Albumin (no. A11A01664) and lactate (no. A11A01721; Horiba Europe GmbH, Hannover, Germany), NEFA (no. 434-91795; Wako Chemicals GmbH, Neuss, Germany), glucose (no. 553-230) and total protein (no. 553-412; MTI Diagnostics GmbH, Idstein, Germany), and urea (no. LT-UR 0050; Labor + Technik E. Lehmann GmbH, Berlin, Germany).

Plasma insulin concentrations were determined using an RIA as previously described (Vicari et al., 2008). Plasma cortisol concentrations were analyzed using a commercial ELISA kit validated for bovine plasma (EIA-1887; DRG Instruments GmbH, Marburg, Germany; Weber et al., 2013). Cross reactivities of the monoclonal antibody against corticosteron and progesterone were 45 and 9 %, respectively, and to any further plasma steroids lower than 2 %. Test sensitivity was 3.4 ng/mL; inter- and intraassay variations were 5.3 and 12.1 %, respectively.

Colostrum was analyzed for DM, CP, crude fat, and ash content (Qualitätsprüfungs- und Dienstleistungsgesellschaft Mecklenburg-Vorpommern mbH, Güstrow, Germany) according to the Weender standard procedure (Naumann and Bassler, 2004), as shown

in Table 2.1. Chemical composition of milk replacer and concentrates were according to the manufacturers' declarations (Table 2.1).

2.2.4 Calculations and statistical analyses

Quercetin measurements were corrected by baseline concentration (first blood sample, before QA or RU administration), then area under the curve (AUC) was calculated for quercetin and its metabolites (isorhamnetin, tamarixetin, and kaempferol) according to the trapeziodale rule between 0 and 24 h using GraphPad Prism 3 (GraphPad Software Inc., San Diego, CA, version 3.03). For single quercetin metabolites, AUC proportion relative to AUC of total flavonols (sum of quercetin, isorhamnetin, tamarixetin, and kaempferol) was calculated in addition. Maximum plasma flavonol concentrations (C_{max}) and time after intake until C_{max} was reached (T_{max}) were determined from individual plasma concentration-time curves. Relative BV of quercetin from RU was calculated as

$$\text{Relative BV} = AUC_{RU}/AUC_{QA} \times 100\ \%.$$

Statistical analyses were performed using SAS/STAT software (SAS Institute, 2010). All data are presented as LSM ± SE. Body weight, concentrate intake, and pharmacokinetic parameters (AUC, C_{max}, T_{max}) as well as proportions of single metabolites on d 2 and 29, were analyzed by repeated measurement ANOVA using the Mixed procedure with group (levels: CTRL, QA, RU; for proportional calculations only QA and RU), repeated variable day (levels: d 2, 29), and group × day interaction as fixed effects in the model. Repeated measures on each calf were taken into account by using the repeated statement of the Mixed procedure with an autoregressive residual covariance structure. Plasma concentration of total flavonols as well as metabolite and hormone data were analyzed by repeated measurement ANOVA using the Mixed procedure, as described previously, with group (levels: CTRL, QA, RU), day (levels: d 2, 29), time changes within d 2 and 29 (several time points before and after feed intake on d 2 and 29, respectively), and respective interactions as fixed effects. Pairwise differences among treatments and time changes were tested by the Tukey-Kramer-test. Effects were considered significant if $P < 0.05$.

2.3 Results

2.3.1 Body weight and feed intake

All calves were born spontaneously and healthy. Birth weight was 47.4 ± 1.0 kg. Body weight increased with time in all groups ($P < 0.01$) and was 65.5 ± 1.3 kg on d 29 without differences between groups. Milk intake was 3.8 ± 0.1 kg/d on d 1 (8 % of BW), 4.8 ± 0.1 kg/d on d 2 (10 % of BW), and 6.9 ± 0.1 kg on d 29. Concentrate intake was 1.59 ± 0.28 kg/calf and increased equally with time ($P < 0.01$) in all groups. Average daily gain from birth to d 29 was 612 ± 28 g/d without group differences.

2.3.2 Bioavailability studies

Plasma concentrations of total flavonols of CTRL were close to or below the detection limit on d 2 and 29, respectively (Figure 2.1A, B). In QA- and RU-fed calves plasma concentrations of total flavonols increased ($P < 0.05$) after flavonoid administration on d 2 and returned to baseline concentrations 48 h after administration (Figure 2.1A).

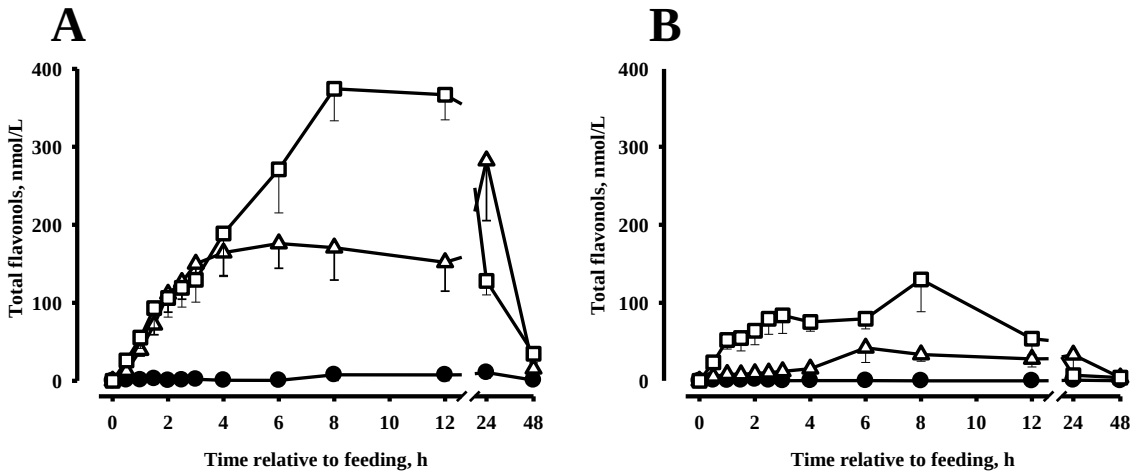

Figure 2.1. Plasma concentration-time-curves of total flavonols after oral administration of 9 mg of quercetin/kg of BW × d as quercetin aglycone (□), rutin trihydrate (△), or no flavonoid (●) on d 2 (A) and 29 (B) of life. Values are LSM ± SE, n = 7.

On d 2, plasma concentrations of total flavonols were greater at 8 and 12 h ($P < 0.01$), but were smaller at 24 h after flavonoid administration ($P < 0.01$) in QA- than in RU-fed calves. On d 29, postprandial plasma concentrations of total flavonols increased far less distinctly ($P < 0.01$) when compared to d 2 (Figure 2.1A, B). Further, plasma concentrations of total flavonols increased until 8 h after administration ($P < 0.01$) in

QA-, but not in RU-fed calves (Figure 2.1B). Plasma concentrations of total flavonols on d 29 tended to be greater ($P < 0.1$) in QA- than in RU-fed calves.

Compared with QA- (100 %), RU-fed calves showed a relative BV of quercetin of 72.5 % on d 2 and of 49.6 % on d 29. On d 2, AUC of total flavonols, quercetin, and quercetin metabolites in QA- and RU-fed calves differed ($P < 0.05$) from CTRL, and were greater ($P < 0.001$) on d 2 than on 29 for both treatments (Table 2.2). On d 2, AUC of quercetin and isorhamnetin was greater ($P < 0.05$) and tended to be greater for total flavonols ($P < 0.1$), but was lower for tamarixetin ($P < 0.05$) in QA- than in RU-fed calves. On d 29, AUC of quercetin was greater (P < 0.05) in QA-fed calves than in CTRL.

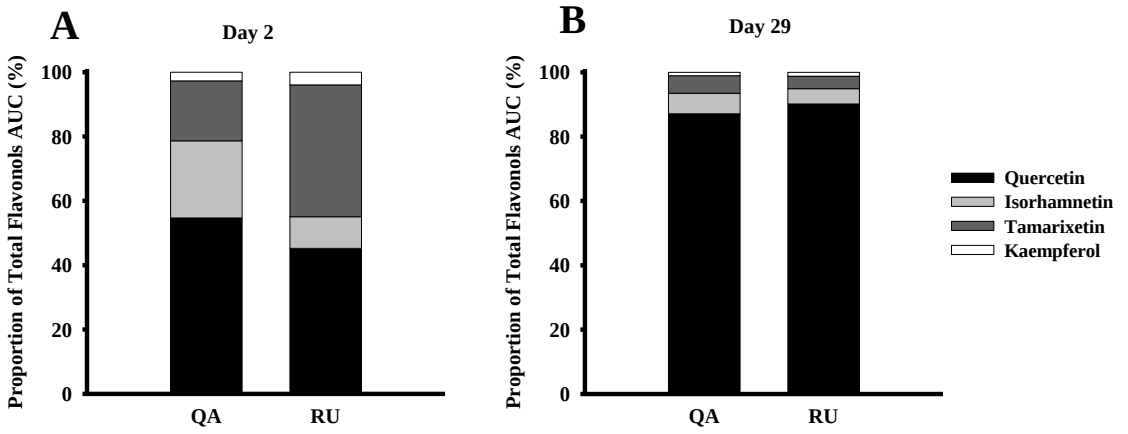

Figure 2.2. Proportion of single flavonols (quercetin, isorhamnetin, tamarixetin, and kaempferol) in plasma after oral administration of 9 mg of quercetin/kg of BW × day as quercetin aglycone (QA) or rutin trihydrate (RU) on d 2 and 29 of life. Values are LSM ± SE, n = 7.

On d 2, C_{max} of total flavonols, quercetin, and quercetin metabolites except kaempferol of QA- and RU-fed calves differed from CTRL ($P < 0.05$; Table 2.2), whereas on d 29 only C_{max} of total flavonols and quercetin of QA-fed calves differed ($P < 0.05$) from CTRL. Maximal plasma concentrations of total flavonols, quercetin, and isorhamnetin decreased ($P < 0.05$) or tended to decrease (kaempferol, tamarixetin; $P < 0.1$) in QA- and RU-fed calves from d 2 to 29, respectively. On d 2, C_{max} was greater ($P < 0.05$) for quercetin and isorhamnetin, but was lower for tamarixetin in QA- than in RU-fed calves. Time to reach C_{max} in QA- and RU-fed calves was not affected by day, but indicated treatment effects for total flavonols, isorhamnetin, and tamarixetin (Table 2.2). For isorhamnetin, T_{max} was greater ($P < 0.05$) in RU- than in QA-fed calves on both days.

Table 2.2. Pharmacokinetic parameters after oral administration of quercetin as quercetin aglycone (QA) or rutin trihydrate (RU) on d 2 and 29 of life; calves of the control group (CTRL) received no flavonoids.

Item[1]	Day	Group[2]			SE	ANOVA (*P*-value)		
		CTRL	QA	RU		Group	Day	Group × day
AUC [3], nmol/L × h								
AUC_{Total}	2	154[c]	5947[a]	4312[ab]	395	0.001	0.001	0.001
	29	7.70	1336	662				
AUC_Q	2	102[c]	3413[a]	1967[b]	228	0.001	0.001	0.001
	29	26.7[b]	1090[a]	535[ab]				
AUC_K	2	31.9[b]	152[a]	159[a]	24.4	0.02	0.001	0.05
	29	0.00	11.1	6.39				
AUC_I	2	0.00[c]	1390[a]	447[b]	70.1	0.001	0.001	0.001
	29	0.00	67.5	239				
AUC_T	2	0.00[c]	1070[b]	1768[a]	154	0.001	0.001	0.001
	29	0.00	58.3	19.6				
C_{max} [4], nmol/L								
$C_{max, Total}$	2	13.9[b]	407[a]	330[a]	35.9	0.001	0.001	0.001
	29	2.24[b]	168[a]	74.7[ab]				
$C_{max, Q}$	2	10.7[c]	238[a]	129[b]	19.3	0.001	0.001	0.03
	29	6.28[b]	137[a]	60.3[ab]				
$C_{max, K}$	2	1.96[b]	12.4[a]	10.9[ab]	2.07	0.01	0.001	0.16
	29	0.00	3.86	2.12				
$C_{max, I}$	2	0.00[c]	94.5[a]	39.6[b]	5.85	0.001	0.001	0.001
	29	0.00	15.5	3.99				
$C_{max, T}$	2	0.00[c]	77.1[b]	163[a]	15.6	0.001	0.001	0.001
	29	0.00	9.45	3.27				
T_{max} [5], h								
$T_{max, Total}$	2	9.50	10.6	16.4	2.84	0.05	0.12	0.90
	29	6.49	6.21	10.4				
$T_{max, Q}$	2	6.65	8.86	11.7	2.77	0.31	0.47	0.76
	29	6.44	5.93	11.1				
$T_{max, K}$	2	10.0	9.67	12.3	4.01	0.93	0.49	0.76
	29	ND[6]	8.00	8.00				
$T_{max, I}$	2	ND	11.4[b]	19.3[a]	2.56	0.001	0.88	0.09
	29	ND	5.90[b]	24.0[a]				
$T_{max, T}$	2	ND	11.1	16.6	3.57	0.01	0.63	0.18
	29	ND	7.63	24.0				

[a-c]LSM in a row with different superscripts differ ($P < 0.05$).
[1]Values are LSM with SE, n = 7 per group.
[2]Group: QA = 30 µmol/(kg BW × d) quercetin aglycone, RU = 30 µmol/(kg BW × d) rutin trihydrate, CTRL = no flavonol fed.
[3]AUC = area under the plasma concentration-time curve of flavonols from 0 to 24 h, calculated by subtracting baseline values (time point 0) from all other time points according to the trapezoidale rule; AUC_{Total}, AUC_Q, AUC_K, AUC_I, AUC_T = AUC of total flavonols (Total) or of the respective quercetin metabolites quercetin (Q), kaempferol (K), isorhamnetin (I), and tamarixetin (T), respectively.
[4]C_{max} = maximum plasma concentration of flavonols subtracted by basal plasma concentration before feed intake; $C_{max, Total}$, $C_{max, Q}$, $C_{max, K}$, $C_{max, I}$, $C_{max, T}$ = maximum plasma concentration of total flavonols (Total), quercetin aglycone (Q), kaempferol (K), isorhamnetin (I), and tamarixetin (T), respectively.
[5]T_{max} = time until C_{max} was reached; $T_{max, Total}$, $T_{max, Q}$, $T_{max, K}$, $T_{max, I}$, $T_{max, T}$ = time until $C_{max, Total}$, $C_{max, Q}$, $C_{max, K}$, $C_{max, I}$, $C_{max, T}$ was reached, respectively.
[6]ND = Not definable.

Relative distribution of single flavonols (quercetin, isorhamnetin, tamarixetin, and kaempferol) proportionately to total flavonols in plasma showed highest values for quercetin on both days and in both groups (QA and RU). Proportion of single flavonols changed with time, namely increased for quercetin ($P < 0.05$) but decreased for isorhamnetin and tamarixetin ($P < 0.05$) from d 2 to 29 (Figure 2.2A, B). On d 2, proportion of isorhamnetin was greater ($P < 0.05$) and proportion of tamarixetin was smaller ($P < 0.05$) in QA- than in RU-fed calves (Figure 2.2A).

2.3.3 Plasma concentrations of metabolites and hormones

Plasma concentrations of total protein decreased ($P < 0.05$) from d 2 to 29 in CTRL and were greater on d 2 in CTRL than in QA-fed calves ($P < 0.05$; Table 2.3). Plasma concentrations of albumin increased ($P < 0.01$) and plasma concentrations of lactate and urea decreased ($P < 0.01$) from d 2 to 29 without any group effect (Table 2.3).

Table 2.3. Blood plasma concentrations of metabolites and hormones of calves after oral administration of quercetin as quercetin aglycone (QA), rutin trihydrate (RU), or no flavonoid (CTRL) on d 2 and d 29.

Item[1]	Day	Group[2]			SE	ANOVA (*P*-value)		
		CTRL	QA	RU		Group	Day	Group × day
Total protein, g/L	2	64.0[a]	59.9[b]	61.7[ab]	0.98	0.03	0.001	0.07
	29	58.0	56.5	59.6				
Albumin, g/L	2	22.9	22.6	22.5	0.37	0.95	0.001	0.42
	29	26.1	26.5	26.3				
Lactate, mmol/L	2	1.68	2.37	2.65	0.27	0.31	0.001	0.04
	29	0.63	0.68	0.69				
Glucose, mmol/L	2	7.37	7.14	7.61	0.28	0.35	0.001	0.99
	29	6.58	6.40	6.79				
NEFA, µmol/L	2	298	281	298	13	0.87	0.001	0.65
	29	172	177	173				
Urea, mmol/L	2	3.80	3.75	3.55	0.26	0.91	0.001	0.82
	29	1.55	1.56	1.55				
Insulin, µg/L	2	2.38	1.79	2.41	0.65	0.12	0.08	0.29
	29	4.38	2.09	2.77				
Cortisol, nmol/L	2	67.2	62.9	77.9	7.0	0.43	0.001	0.67
	29	11.1	8.6	13.0				

[a-b]LSM in a row with different superscripts differ ($P < 0.05$).
[1]Values are LSM based on plasma concentrations in blood samples taken before and until 24 h after morning feeding on d 2 and 29, respectively; n = 7 per group.
[2]Group: QA = 30 µmol/(kg BW × d) quercetin aglycone; RU = 30 µmol/(kg BW × d) rutin trihydrate; CTRL = no flavonol fed.

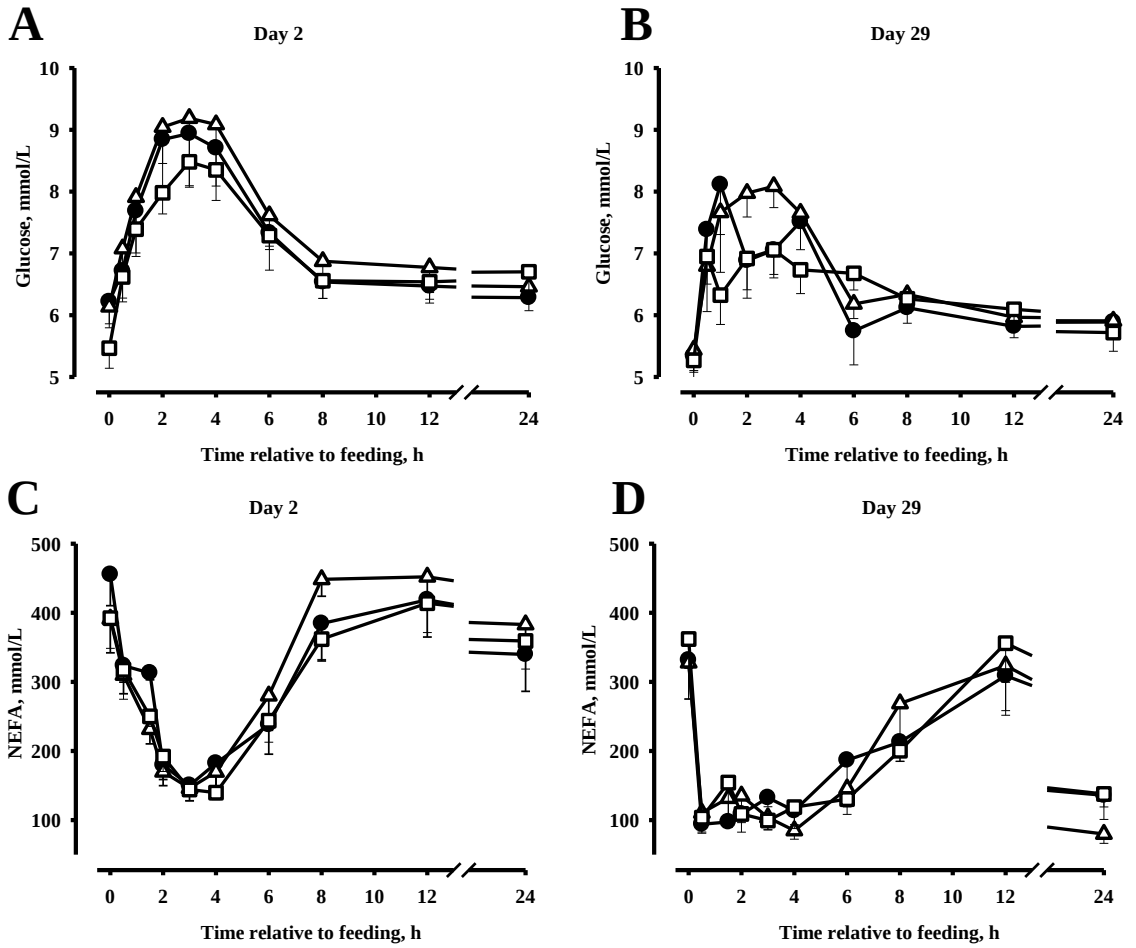

Figure 2.3. Plasma concentrations of glucose (A, B) and NEFA (C, D) after oral administration of 9 mg of quercetin/kg BW × d as quercetin aglycone (□), rutin trihydrate (△), or no flavonoid (●) on d 2 and d 29 of life. Values are LSM ± SE; n = 7.

Mean glucose and NEFA plasma concentrations decreased ($P < 0.05$) from d 2 to 29 in all groups, but concentrations did not differ among groups (Table 2.3). Considering the day course of the plasma metabolites, plasma concentrations of glucose increased ($P < 0.01$), whereas NEFA decreased ($P < 0.01$) after feed intake in all groups on d 2 and 29, respectively (Figure 2.3A-D).

The increase of plasma insulin concentrations after feed intake was more pronounced ($P < 0.05$) on d 29 than on d 2, but without group effects (Figure 2.4A, B; Table 2.3). Plasma concentrations of cortisol decreased ($P < 0.05$) after feed intake on both days with mean concentrations being greater ($P < 0.05$) on d 2 than on d 29 (Figure 2.4C, D; Table 2.3).

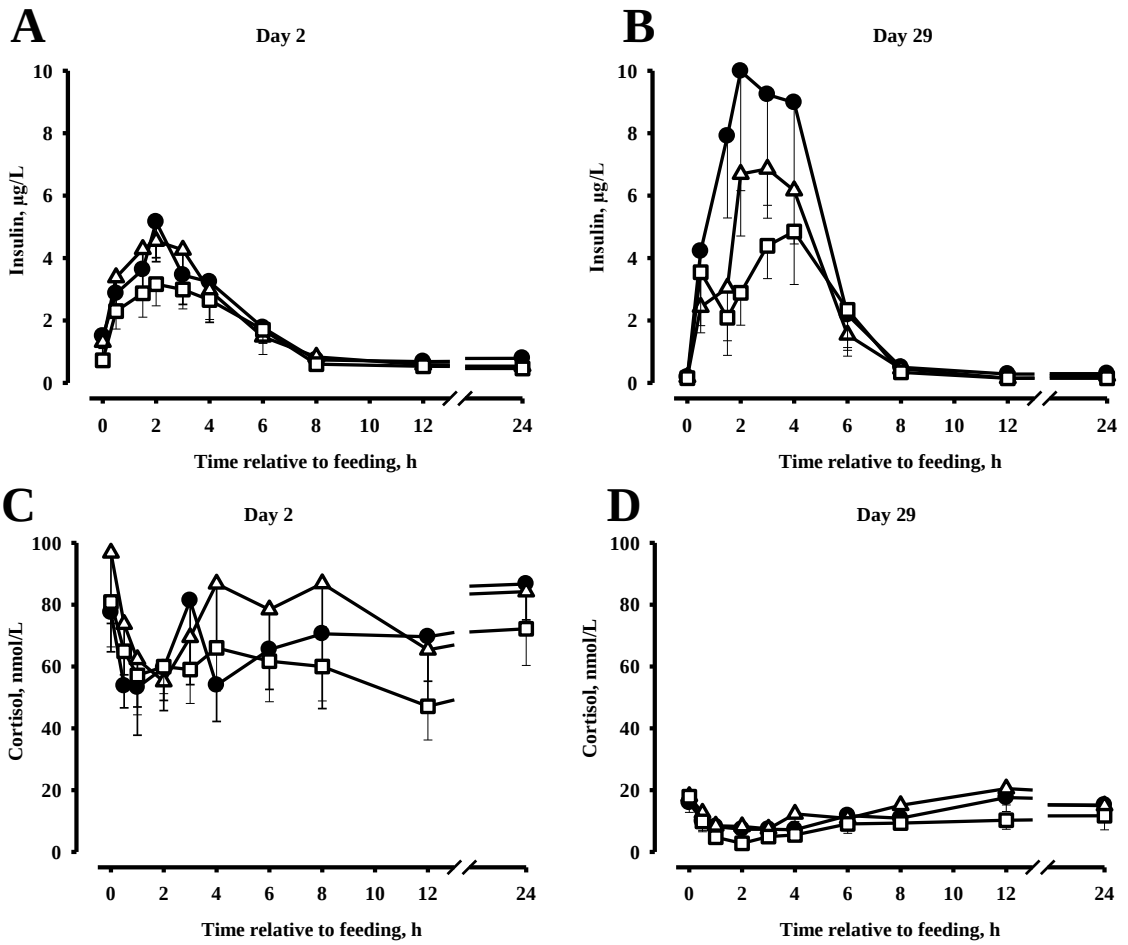

Figure 2.4. Plasma concentrations of insulin (A, B) and cortisol (C, D) after oral administration of 9 mg of quercetin/kg of BW × d as quercetin aglycone (□), rutin trihydrate (△), or no flavonoid (●) on d 2 and d 29 of life. Values are LSM ± SE, n = 7.

2.4 Discussion

Without dietary supplementation of quercetin (CTRL group) plasma concentrations of total flavonols were close to or below the detection limit on d 2 and 29. However, small amounts of quercetin and its dehydroxylated metabolite kaempferol were found in blood plasma on d 2 and 29 in the control group. According to Besle et al. (2010), flavonoids are natural components of bovine milk and their concentration is affected by cow's diet. Although colostrum and milk replacer were not analyzed for flavonoid content in the present study, it can be assumed that colostrum is most likely the source for flavonoids found in CTRL calves at d 2. Apart from colostrum, feed components such as concentrates and hay may also contain flavonoids (Fraisse et al., 2007; Reynaud et al., 2010), which could explain measurable, albeit very low, plasma concentrations of quercetin and its metabolites at least in older calves (d 29).

In calves, quercetin was systemically available from both supplements, QA as well as RU. Interestingly, plasma values obtained for quercetin and its metabolites with an intact flavonol structure were greater on d 2 than on d 29, indicating better absorption or less excretion in younger animals. This may be explained by a different degree of gastrointestinal maturation and intestinal permeability on d 2 and 29, respectively (Blum, 2006). Thus, the epithelium in newborn animals is characterized by vacuolated epithelial cells which are not present in older animals (Bainter, 2002; Blum, 2006). This has possibly contributed to a greater absorption of quercetin from both sources (QA and RU) on d 2 compared with d 29 in calves. Furthermore, the reticulorumen and microbial activities might be already more developed in 29- than in 2-d-old calves, resulting in partial ruminal degradation of the flavonoids applied (Heinrichs and Jones, 2003; Guilloteau et al., 2009; Berger et al. 2012; Li et al., 2012). The BV of quercetin from both, QA and RU, especially on d 29 could probably be improved when encapsulated quercetin preparations will be used (Ding et al., 2014).

On both days investigated, feeding of QA resulted in greater plasma concentrations of total flavonols, quercetin, and its metabolites than feeding of RU, indicating QA as a better source of quercetin than RU with respect to the BV of quercetin. Markedly lower plasma concentrations of total flavonols were also seen in monogastric species after feeding of RU instead of QA at dosages comparable to the current study (Manach et al., 1997; Cermak et al., 2003; Reinboth et al., 2010). Thus, the present results obtained in calves are in principal accordance with studies on the BV of quercetin in monogastric species such as rats (Manach et al., 1997), dogs (Reinboth et al., 2010), pigs (Cermak et al., 2003; Lesser et al., 2004), and humans (Erlund et al., 2000; Egert et al., 2008). As the newborn calf is considered as a functionally monogastric animal (Drackley, 2008), these findings are not surprising.

Concerning RU application, studies in monogastric species often indicate a delayed increase of quercetin and its metabolites in blood plasma (Erlund et al., 2000; Cermak et al., 2003; Reinboth et al., 2010). In the present study, maximal plasma concentrations of quercetin and its metabolites were measured much later after RU than after QA feeding, indicating different intestinal sites for absorption (Erlund et al., 2000). In RU-fed calves, quercetin and its metabolites, at least on d 2, most likely derive from absorption in both the small and the large intestine. In addition, we found the same slow increase of quercetin and its metabolites in blood plasma after QA and RU feeding, but the increase

stopped much earlier in RU- than in QA-fed calves. In general, the sugar moiety in flavonol molecules determines the absorption of flavonoids in organisms (Day et al., 1998; Hollman et al., 1999) and QA is supposed to be already absorbed in the small intestine. More complex glycosides, such as the glucorhamnoside RU, are absorbed in distal parts of the small intestine or in the colon of monogastric animals, where flavonol molecules are hydrolyzed by microorganisms. Thus, the lack of rhamnosidase activity in the small intestine can be considered as one reason for delayed and lower absorption of RU than of QA (Erlund et al., 2000; Cermak et al., 2003). Our pattern of relative BV of total flavonols after RU feeding fits to relative BV of total flavonols in blood plasma after duodenal, but not after ruminal application in dairy cows (Berger et al., 2012; Gohlke et al., 2013), indicating a low ruminal function in our calves during first month of life.

Interestingly, pharmacokinetics of quercetin absorption after QA feeding markedly differed between calves and adult cattle (Berger et al., 2012; Gohlke et al., 2013) or monogastric animals (Erlund et al., 2000; Cermak et al., 2003; Reinboth et al., 2010). The typical peak of total flavonol plasma concentrations some minutes after QA administration was not seen in newborn calves. As a consequence, time for reaching C_{max} was much greater in calves than in adult ruminants and monogastrics. Comparable slow increases of quercetin and its metabolites as in newborn calves' blood plasma were only seen in rats (Manach et al., 1997). These differences in pharmacokinetics after QA feeding are difficult to explain, but a probable reason is the low release of milk protein into the duodenum due to abomasal casein clotting (Heinrichs and Jones, 2003; Guilloteau et al., 2009) and the potential binding of flavonoids to milk proteins (Gugler et al. 1975; Boulton et al., 1998; Janisch et al., 2004).

All measured metabolites and hormones were in physiological ranges and most of these findings were in accordance with previous results indicating postnatal growth, especially protein accretion, and development in breeding as well as suckling calves (Egli and Blum, 1998; Nussbaum et al., 2002; Schiessler et al., 2002). Conversely, except for total protein concentrations on d 2, we found no treatment effects by QA or RU feeding in these calves. Plasma protein concentrations were lower in QA-fed than in CTRL calves, a finding that was hard to explain because all calves received the same pooled colostrum with identical IgG content at first days of life. Postprandial changes of metabolites and hormones have been reported previously (Hadorn et al., 1997; Hammon

and Blum, 1998), but systemic glucose metabolism was not affected by QA or RU feeding, as we have assumed from literature (Cermak et al., 2004; Tadera et al., 2006).
In conclusion, BV of quercetin and its metabolites in newborn calves is more pronounced when quercetin is fed as QA than as RU. These findings go along with data on BV of quercetin in lactating cows with duodenal application (Gohlke et al., 2013), and in monogastric species like rats, dogs, and pigs (Manach et al., 1997; Cermak et al., 2003; Reinboth et al., 2010). Greater BV of quercetin on d 2 than on d 29 of life was probably due to reduced maturation status of the gastrointestinal tract in 2-d old calves. However, effects of quercetin feeding on the antioxidative status should be investigated to validate health-protecting effects of quercetin in neonatal calves.

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Chapter 3

Effects of oral flavonoid supplementation on metabolic and antioxidative status in newborn dairy calves

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3. Effects of oral flavonoid supplementation on metabolic and antioxidative status in newborn dairy calves

Abstract

Many studies investigated effects of natural feed additives like flavonoids and verified their antioxidative or antiinflammatory properties, but scientific proof for flavonoids as health and performance-promoting tool in calf nutrition is weak. Thus, we investigated the effects of the most abundant flavonoid quercetin and of a green tea extract (GTE) containing various catechins on metabolic and antioxidative traits in dairy calves to further clarify potential health-promoting effects. Male newborn German Holstein calves (n=7 per group) either received no flavonoid (control group), 10 mg of quercetin equivalents as quercetin aglycone or as rutin/kg of body weight (BW) and d, or 10 mg/kg BW and d of a GTE from d 5 to d 26 of life with morning and evening feeding. All calves were fed equal amounts of colostrum and milk replacer according to BW. Body Weight, feed intake, and health status were evaluated daily. Blood samples were collected from the jugular vein on d 1, 5, 12, 19, and 26 before morning feeding to investigate flavonoid, metabolic, and antioxidative status in calves. During the experiment, growth performance data and health status remained unchanged, but GTE-fed calves had less loose feces than controls and calves fed quercetin aglycone required less medical treatment than other groups. Concentrations of quercetin changed over time and were higher in rutin-fed than control group, whereas catechins were below detection limit. Plasma trolox equivalent antioxidative capacity (TEAC) and ferric reducing ability of plasma (FRAP) were measured as markers for plasma antioxidative capacity. Concentrations of TEAC increased whereas FRAP decreased after the first d of life in all groups, least in controls. Oxidative stress markers in plasma were measured as thiobarbituric acid reactive substances (TBARS) and F2-isoprostances. Concentrations of TBARS were highest during first month of life in the control group, but decreased at the same time in the GTE group. F2-isoprostane concentrations decreased in control group only. Plasma concentrations of total protein, albumin, urea, lactate, glucose, and non-esterified fatty acids as well as of insulin and cortisol varied in time, but there were no group differences caused by flavavonoid supplementation. Taken together, orally administrated quercetin and catechins at dosages used in the

present study resulted in no or only weak effects on health, metabolic, and antioxidative status in newborn dairy calves.

Key Words: antioxidative status, calf, flavonoid, quercetin, rutin

3.1 Introduction

After birth, calves undergo huge immunological and metabolic changes. Though relatively mature at birth, they need to adapt to various morphological and functional changes after birth (Blum and Hammon, 2000). Calf losses mainly occur during first wk of life, mostly because of respiratory and digestive problems (USDA, 2011). Good management, like early and sufficient colostrum supply is essential. In addition, fed supplements are daily practice in modern dairy. Particularly since the ban of antibiotic growth promoters in the European Union in 2006, the call for 'natural' feed additives gained enormous popularity. In this respect, flavonoids as secondary plant metabolites, ubiquitous in all higher plants are of interest. Their health-promoting properties are mainly thought to be due to their strong antioxidant activity *in vitro*, associated with effects on several metabolic key enzymes and inflammatory cells (Middleton et al., 2000), glucose and lipid metabolism (Shetty et al., 2004; Kobayashi et al., 2010) or incidence of diarrhea (di Carlo et al., 1994; Gálvez et al., 1995). One of the most abundant flavonoids is the flavonol quercetin, a pentahydroxyflavon mostly bound β-glycosidic manner to at least one sugar molecule and present in high concentrations in apples and onions. Quercetin bound to rutinose is called rutin and is the major glycoside of quercetin. The predominant flavanol, another subgroup of flavonoids, is catechin, which is mainly found in high concentrations in green tea.

Almost any disease is associated with increased formation of reactive oxygen species, thus causing oxidative stress (Halliwell, 1991). In calves, enhanced oxidative stress was seen on the first d of life (Alexandrovich and Antonovna, 2009) and especially if sick (Ahmed and Hassan, 2007; Al-Qudah, 2009). Because the nutrient status often is impaired in newborn calves, this possibly affects the antioxidant system (Schwerin et al., 2002; Sies et al., 2005). Thus, improvement of the antioxidant status in newborn calves may accelerate maturation of the own immune system, improve health status, and thus reduce calf losses. The bioavailability of quercetin in newborn calves as prerequisite for biological effects was recently shown (Maciej et al., 2015). Based on these findings we have tested the hypothesis that quercetin and catechin

supplementation affect metabolism and the antioxidative and health status during the first 3 wk of life in dairy calves.

3.2 Materials and Methods

3.2.1 Animals and feeding

Procedures performed in this study were in accordance with the German animal protection law and approved by the relevant authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; permission no. LVL M-V/TSD/7221.3-2.1-019/10). Twenty-eight male German Holstein calves were examined from d 1 to d 26 of life. All calves were spontaneously born from multiparous cows on neighboring farms and transported directly after birth to the experimental barn of the University of Rostock, where they were kept in single boxes with straw bedding. Calves had free access to water and were fed twice daily by nipple bottle or nipple bucket. On the first 3 d of life calves received pooled colostrum obtained from milkings 1, 3, and 5 (d 1, 2, and 3 after parturition, respectively) in amounts of 8 % of BW on d 1 and 10 % of BW on d 2 and 3 (Table 1). From d 4 until d 26 calves received a commercial milk replacer (150 g/L; SalvaLac MiraPro 45, Salvana Tiernahrung GmbH, Klein-Offenseth Sparrieshoop, Germany) in amounts of 12 % of BW/d. To ensure equal feed uptake in all groups refused amounts of colostrum or milk replacer were tub- fed. Milk replacer amounts were adapted to BW once a wk.

From d 2 to d 6, colostrum or milk replacer was supplemented with chicken-egg derived immunoglobulins (Globigen Life Start 25 %, EW Nutrition GmbH, Visbek, Germany) fed twice daily in amounts of 40, 32, 24, 16, and 8 g/d, respectively (Maciej et al., 2015).

Calves had free access to pelleted concentrate (Kälber Start 18/3 pell., Vollkraft Mischfutterwerke GmbH, Karstädt, Germany) and hay from d 4 on. Concentrate intake was measured daily after the morning milk feeding (Maciej et al., 2015).

Table 3.1. Composition and amounts of colostrum, milk replacer, and concentrate fed to calves.

Item	d 1 colostrum[1] milking 1	d 2 colostrum[1] milking 3	d 3 colostrum[1] milking 5	d 4 to 30[2] Milk replacer[3]	d 4 to 30[2] Concentrate[4]
DM, g/kg	206.9	154.7	139.3	110.0	880.0
CP, g/kg DM	597.4	390.4	333.1	201.8	180.0
Crude fat, g/kg DM	169.2	240.5	274.9	190.9	43.0
Ash, g/kg DM	48.3	55.6	56.0	73.6	74.0
Lactose, g/kg DM	136.3	260.5	308.7	514.6	-

[1]Colostrum was derived from multiparous cows and separately pooled from milkings 1, 3, and 5 after parturition, respectively.
[2]Hay was offered ad libitum except for days of bioavailability studies.
[3]Milk replacer (SalvaLac MiraPro 45, Salvana Tiernahrung GmbH, Klein-Offenseth Sparrieshoop, Germany), was composed of 45 % spray skimmed milk powder, 35 % sweet whey powder, 18 % plant oil (palm, coconut and soy oil), 1.7 % Lys, 0.8 % Ca, 0.75 % P, and added with 50,000 IU of vitamin A, 4,000 IU of vitamin D_3, 200 mg of vitamin E (α-tocopherol acetate), 9 mg Cu (glycerine-copper chelate), citric acid, sorbic acid, butylhydroxytoluene (BHT), and 1.2 x 10^9 cfu *Enterococcus faecium* E1706.
[4]Concentrate (Kälber Start 18/3, Vollkraft, Mischfutterwerke GmbH, Karstädt, Germany) was composed of wheat gluten, wheat bran, grain mash, sugar beet pulp, rapeseed meal, oat, rye, molasses, linseed, oat bran, soybean meal extract, calcium carbonate, sodium chloride, 0.6 % phosphorous, 0.2% sodium, 10,800 IU of vitamin A, 1,215 IU of vitamin D_3, 30 mg of vitamin E, 0.7 mg of I, 0.4 mg of Co, 54 mg of Mg, 81 mg of Zn, and 0.4 mg of Se per kg of DM.

Health status of calves was determined daily by measuring rectal temperature, heart and respiratory rate, by evaluation of behavioral abnormalities, nasal discharge, respiratory sounds, and by navel inspection. Fecal consistence was assessed daily by fecal consistence score according to Larson et al. (1977): normal (1), soft (2), runny (3), or watery (4). Sick calves were treated by a veterinarian; for statistical analysis each calf was counted as treated for a disease independent from how many times an animal was treated.

3.2.2 Treatment and blood sampling

Calves were randomly assigned to one of four feeding groups (n = 7). Control (**CTRL**) received no flavonoids, **QA** received 10 mg/kg BW × d quercetin aglycone (quercetin dihydrate; Carl Roth GmbH & Co. KG, Karlsruhe, Germany), **RU** received 20 mg/kg BW × d quercetin as glucorhamnoside rutin (rutin trihydrate; Carl Roth GmbH & Co. KG), and **CA** received 10 mg/kg BW × d of a green tea extract (**GTE**) containing various catechins (Polyphenon 60; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). For QA and RU, daily dose of quercetin equivalents was 10 mg/kg of BW (30 µmol/kg BW). The GTE (70.3 % total catechins) fed in CA was composed of 1.4 % catechin, 0.3 % catechin gallate, 5.2 % gallocatechin, 2.1 % gallocatechin gallate (all as trans-isomers) and 6.4 % epicatechin, 7.0 % epicatechin gallate, 19.0 % epigallocatechin, 28.8 % epigallocatechin gallate (all as cis-isomers). Amounts of flavonoid fed to calves in this study were based on previous studies in pigs and dogs (Lesser et al., 2004; Reinboth et al., 2010; Lühring et al., 2011). Due to studies on bioavailability of flavonoids, all calves received their respective daily dose with the morning feeding on d 2, and no flavonoids were fed on d 3 and 4. From d 5 on, the daily dose was equally split with morning and evening meal since d 26. On d 1, 3, and 4 no flavonoids were fed. Flavonoids were suspended in water and administered with a disposable 10-ml syringe directly into the mouth during milk feeding.

Jugular blood samples were taken immediately after birth, and on d 5, 12, 19, and 26 before morning feeding using evacuated tubes (Vacuette; Greiner Bio-One GmbH, Frickenhausen, Germany). Tubes containing 15 I.U./mL lithium heparinate were used for the determination of the plasma concentrations of flavonols, catechins, and the markers for antioxidative capacity and oxidative stress; tubes containing 2.5 g/L sodium fluoride and 1.8 g/L potassium EDTA were used for the determination of plasma

protein, albumin, glucose, NEFA, urea, and lactate, and tubes containing 1.8 g/L potassium-EDTA were used for the determination of plasma insulin and cortisol concentrations. The blood samples were immediately put on ice and centrifuged (1,500 × *g*, 4°C, 20 min). To measure the catechins 1 mL of plasma was mixed with 20 µL of an ascorbate-EDTA solution (0.4 mol/L NaH_2PO_4; Carl Roth GmbH & Co. KG) containing 20 % ascorbic acid (Merck KGaA, Darmstadt, Germany) and 0.1 % EDTA (Carl Roth GmbH & Co. KG) at pH 3.6. To measure F2-Isoprostanes, a 0.005 % ethanolic butylhydroxytoluene (BHT) solution (0.005 % BHT in ethanol, w/v) was added in one percent proportion for stabilization. The plasma samples were stored until analyzed at -20°C for metabolites and hormones and at -80°C for flavonoid and (anti)oxidative parameters until analyzed.

3.2.3 Analytical methods

Plasma concentrations of quercetin aglycone and its methylated (isorhamnetin and tamarixetin) and dehydroxylated (kaempferol) derivatives were analyzed by HPLC with fluorescence detection as described previously (Berger et al., 2012). The detection limit of flavonol was ≤ 10 nmol/L and the recovery rate of flavonols was 92 ± 2 % (mean ± SEM). Inter- and intraassay coefficients of variability for quercetin were 7.2 and 0.5 %, respectively. The sum of quercetin and its metabolites in plasma is referred to as total flavonols.

Plasma concentrations of individual catechins (gallocatechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate, and epicatechin) were determined by HPLC (Lee et al., 1995) with some modifications (Egert et al., 2013). In brief, 500 µL of thawed plasma were mixed with 37.5 µL of β-glucuronidase/sulfatase (final activities of 7,300 and 130 U/mL for glucuronidase and sulfatase, respectively; Sigma Aldrich Chemie GmbH), and incubated at 37°C for 45 min. Hydrolyzation was stopped by adding 1 mL methylene chloride. Samples were mixed for 4 min and centrifuged (3,220 × *g*, 4°C, 15 min). The supernatant was transferred into another tube, mixed with 1 mL ethyl acetate for 4 min, and centrifuged (3,220 × *g*, 4°C, 20 min); and 800 µL of the supernatant were transferred into a glass tube with glass pearls and ethyl acetate extraction was repeated twice. Supernatants were combined, mixed with 10 µL of 1 % aqueous ascorbic acid, and dried by vacuum centrifugation (SPD2010 SpeedVac System; Thermo Fischer Scientific GmbH, Dreieich, Germany). The dried sample was

dissolved in 150 µL of the mobile phase A by vortex mixing and ultrasonic bath for 15 min. After centrifugation 30 µL of the supernatant were used for HPLC analyses. For catechin analyses by HPLC (Jasco Deutschland GmbH, Groß-Umstadt, Germany; pump model PU-2080 plus), the supernatant was injected into the fully automated autosampler (AS-2057 Plus; Jasco Deutschland GmbH). Separation occurred on a reverse-phase C-18 Kromasil 100 column (25 × 4.6 mm, 5 µm; Jasco Deutschland GmbH) protected by a pre-column (C-18 Inertsil ODS-2, 10 × 4 mm, particle size 5 µm; Jasco Deutschland GmbH) at 30°C. Mobile phase A and B were composed of water, acetonitrile, and trifluoroacetic acid (92:8:0.1 and 65:35:0.1 v/v/v) with a pH of 2.5. At a flow rate of 0.9 mL/min, the eluent was monitored by electrochemical detection with potential settings at 0, 120, 240, and 360 mV in a 4-channel colometric electrochemical detector (Coul Array, 5600A; ESA Inc., Chelmsford, MA). Dominant signals used for quantification of catechins were 120 mV for epigallocatechin and epicatechin gallate, and 240 mV for catechin, gallocatechin, epicatechin, and epigallocatechin gallate, respectively. Quantification of individual plasma catechins were carried out using external standards, which were generated by simultaneously adding catechin, gallocatechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate, and epicatechin (Carl Roth GmbH & Co. KG) to untreated plasma at final concentrations of each catechin of 0.125, 0.25, 0.5, 1, 2.5, 5, 7.5, and 10 µmol/L. Calibration samples were treated in the same way as experimental samples. The coefficient of determination was $r \geq 0.99$, detection limit of catechins was ≤ 10 nmol/L, and the intra-assay coefficient of variation was 2.4 ± 2.6 to 6.5 ± 4.7 %.

Antioxidative capacity and oxidative stress markers were analyzed in repeated measurements. The trolox equivalent antioxidative capacity (TEAC) is defined as amount of the water-soluble vitamin E derivate trolox (in mmol) needed to show the same antioxidant capacity at a defined time point as 1 mmol of plasma and is expressed as trolox equivalents (TE) in mmol/L plasma. The TEAC was measured according to the modified protocol of Re et al. (1999) by measuring spectrophotometrically the decolorization of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{\cdot+}$) caused by binding to antioxidants at a wavelength of 734 nm. Ferric reducing ability of plasma (FRAP) was determined according to Benzie and Strain (1996) and is given in ascorbic acid equivalents (ASCE) in µmol/mL plasma. Reduction of Fe^{3+} - tripyridyltriazin by antioxidants from plasma results in a color change that is directly

proportional to antioxidative capacity. Absorbance was measured at a wavelength of 595 nm and compared to the one of ascorbic acid. Thiobarbituric acid reactive substances (TBARS) concentration in plasma was measured according to Yagi (1998) with modifications and is given as malondialdehyde equivalents (MDAE) in µmol/L plasma. Adding thiobarbituric acid to the sample results in formation of a red complex, whose optical density was measured photometrically at a wavelength of 532 nm. F2-isoprostanes, especially their main representative 8-iso-prostaglandin $F_{2\alpha}$, are produced *in vivo* by peroxidation of the cell membrane lipid component arachidonic acid, and is considered as a reliable marker for lipid peroxidation. Detection of 8-iso-prostaglandin $F_{2\alpha}$ was carried out with an enzymatic immune assay (Direct 8-iso-prostaglandin $F_{2\alpha}$; Assay Designs, MI) and values are expressed in pg/mL.

Plasma metabolites were analyzed by the Clinic for Cattle (University of Veterinary Medicine Hannover, Foundation, Germany) spectrophotometrically on an ABX Pentra 400 (Horiba ABX SAS, Montpellier Cedex, France) using the respective kits: Albumin (no. A11A01664) and lactate (no. A11A01721; Horiba Europe GmbH, Hannover, Germany), NEFA (no. 434-91795; Wako Chemicals GmbH, Neuss, Germany), glucose (no. 553-230) and total protein (biuret reaction, no. 553-412; MTI Diagnostics GmbH, Idstein, Germany), and urea (no. LT-UR 0050; Labor + Technik E. Lehmann GmbH, Berlin, Germany). Plasma insulin and cortisol concentrations were measured either by radioimmunoassay or by ELISA, respectively, as previously described (Maciej et al., 2015).

Colostrum was analyzed for DM, CP, crude fat, and ash content (were measured by MQD Qualitätsprüfungs- und Dienstleistungsgesellschaft Mecklenburg-Vorpommern, Güstrow, Germany) using standard procedures according to Weende (Naumann and Bassler, 2004). Chemical components and contents of the milk replacer were provided by the manufacturer according to Weender analysis (Naumann and Bassler, 2004; Table 3.1).

3.2.4 Statistical analyses

Statistical analyses were performed using SAS software, Version 9.4 for Windows. Copyright, SAS Institute Inc., Cary, NC, USA. Data on health, performance, and from plasma measurements are presented as least squares means (LSM) ± standard error (SE) and were analyzed by repeated measurement ANOVA using the Mixed procedure of

SAS/STAT software with a model containing the fixed effects treatment group (levels: CTRL, QA, RU, CA; for plasma flavonol concentrations only CTRL, QA, RU), day (levels: 1, 5, 12, 19, 26) and group × day interaction. Repeated measures on the same animal were taken into account by the REPEATED statement of the MIXED procedure and an autoregressive type for the block diagonal residual covariance matrix. Pairwise differences among treatment groups and days were tested by the Tukey-Kramer-test. Data on fecal score and medical treatment were analyzed with the NPAR1WAY procedure of SAS/STAT software across the one-way classification treatment group (levels: CTRL, QA, RU, CA) using Wilcoxon scores (for each wk seperately). Effects and differences were considered significant if $P < 0.05$.

3.3 Results

3.3.1. Body weight, feed intake, and health status

All calves were born spontaneously and were evidently healthy. Body weight was 47.3 ± 1.0 kg at birth, and did not differ among groups.

Average daily gain (616 g/d ± 25 g; n = 28) and final BW (58.2 kg ± 0.42 kg, n = 28) was not different between groups.

Milk intake increased with time (wk 1 = 5.1 kg/d; wk 2 = 6.1 kg/d, wk 3 = 6.4 kg/d, wk 4 = 7.0 kg/d; $P < 0.001$) equally in all groups. Concentrate intake also increased with time ($P < 0.001$) equally in all groups. During the first wk concentrate intake was very low (4 g/d), but increased to 27 g/d during the second wk and to 117 g/d in wk 4.

Average fecal score for all groups was 1.4 and each calf had on average 2 d of strong diarrhea, indicated by fecal score 4. Severe diarrhea occurred between d 3 and d 20, and fecal score was highest in wk 2 (fecal score 1.62) and wk 3 (fecal score 1.54). During the first wk of life, fecal score differed among groups, being lower ($P < 0.05$) in CA group (fecal score 1.01) than in CTRL group (fecal score 1.46) and in group QA (fecal score 1.31).

Treatments of diseases showed no significant group differences. However, group CA had 3 treated calves (total 7 treatments: 2 diarrhea, 4 pneumonia, 1 other disease) and other groups 6 treated calves each [CTRL: in total 9 treatments (4 diarrhea, 2 pneumonia, 3 other diseases); RU: in total 10 treatments (1 diarrhea, 7 pneumonia, 2 other diseases); QA: in total 10 treatments (1 diarrhea, 5 pneumonia, 4 other diseases)].

Other diseases were omphalitis (n = 2), thromboflibitis (n = 4), polyartritis (n = 1), obstipation (n = 1), and conjuctivitis (n = 1).

3.3.2 Plasma flavonoid concentrations

Plasma concentrations of total flavonols in QA and RU increased ($P < 0.05$) until d 12, but decreased thereafter in QA (Figure 3.1). Plasma concentrations of total flavonols (sum of quercetin, isorhamnetin, and tamarixetin) were higher ($P < 0.05$) in RU than in CTRL (data for quercetin metabolites not shown).
Plasma concentrations of individual catechins were below detection limit.

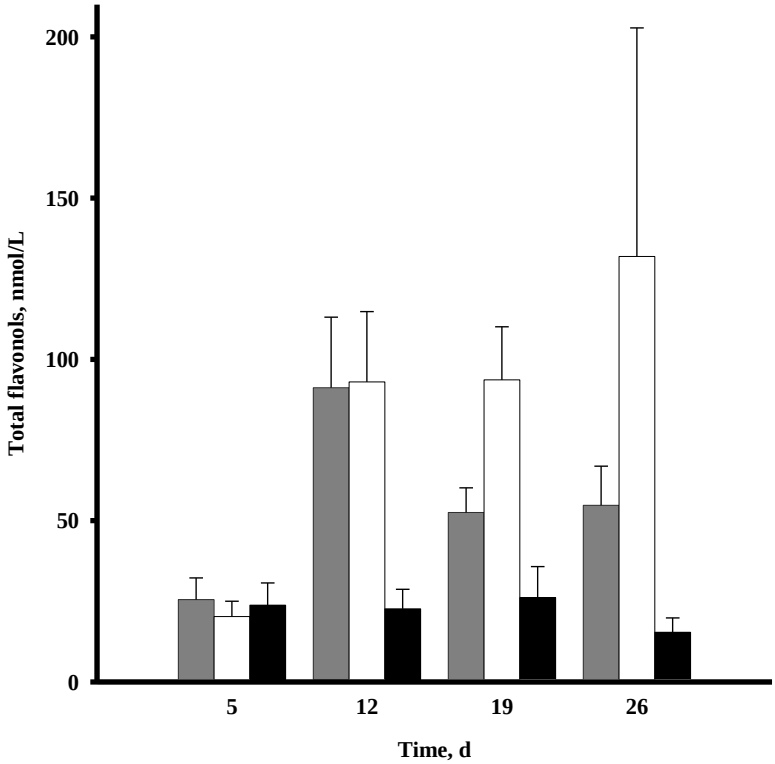


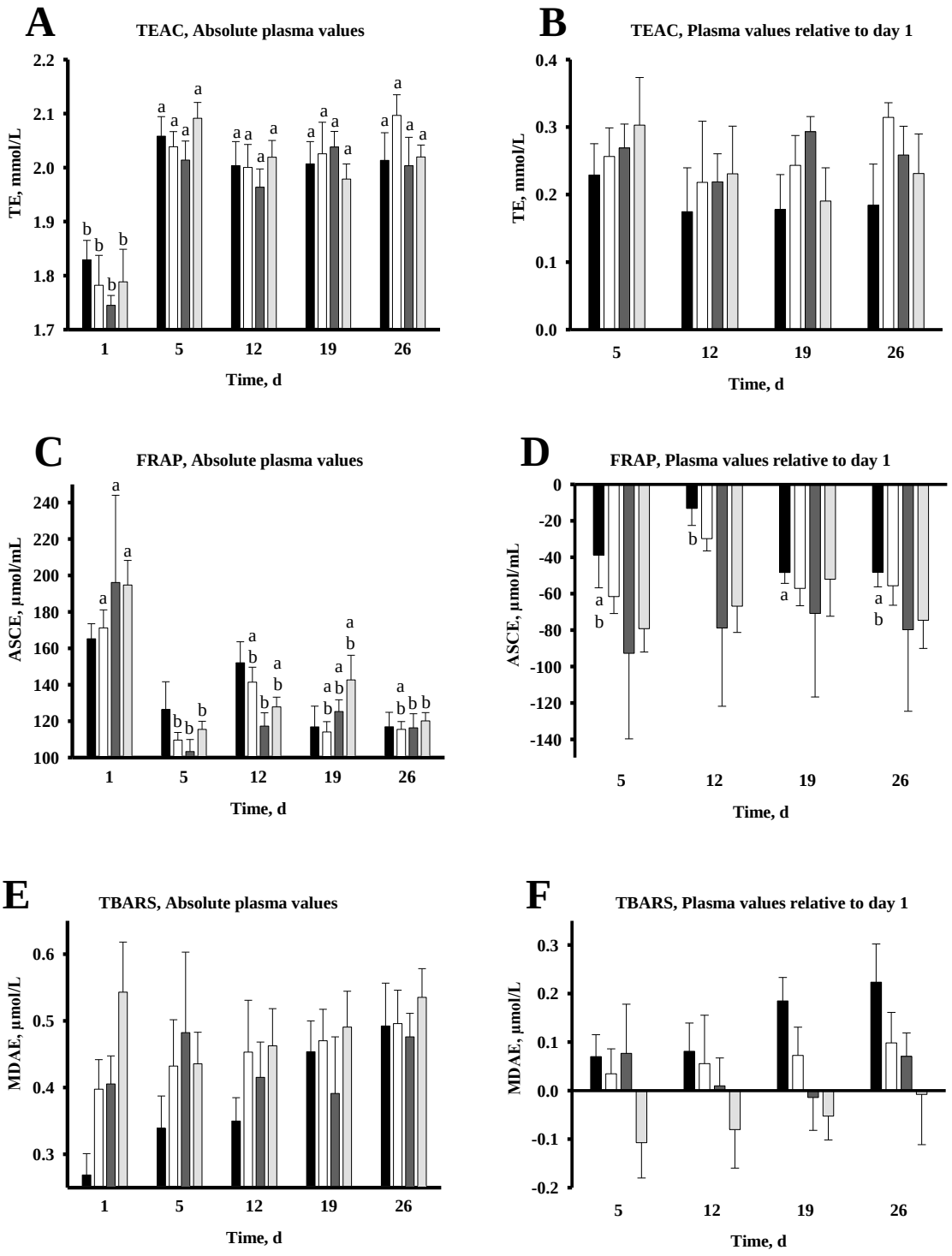
Figure 3.1. Plasma concentrations of total flavonols from d 5 to 26 after feeding of no flavonoid (■) and after oral administration of 9 mg of quercetin/kg of BW × day as quercetin aglycone (■) or as rutin (□); values are LSM ± SE, n = 7 per group.

3.3.3 Markers for antioxidative capacity and oxidative stress

Markers for antioxidative capacity (TEAC, FRAP; Figure 3.2A-D) changed with time, with TEAC increasing and FRAP decreasing from d 1 to d 5 and then remaining relatively stable in all groups. Absolute concentrations as well as relative changes did not differ among groups, but FRAP showed a group × time interaction ($P < 0.001$) for

relative changes (in CTRL from d 12 to d 19; $P = 0.03$ and in RU from d 5 to d 12; $P = 0.08$), but no relative changes in QA and CA.

Absolute concentrations of oxidative stress markers (TBARS, F2-isoprostanes) revealed huge individual variations within groups and did not show group or time differences (Figure 3.2 E, G). However, relative changes of both oxidative stress markers showed overall group effects between CA and CTRL ($P < 0.05$; Figur 3.2 F, H).



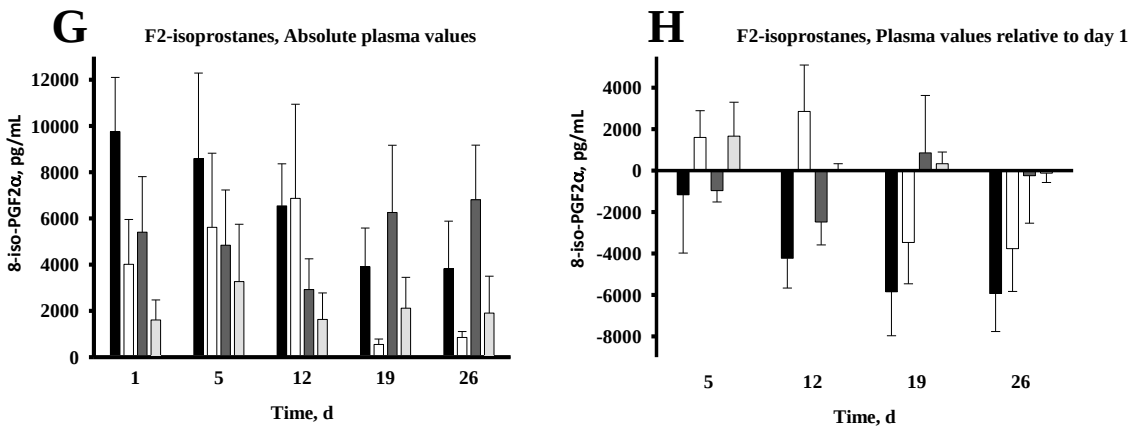


Figure 3.2. Time course of different markers for antioxidative capacity (TEAC = trolox equivalent antioxidative capacity, FRAP = ferric reducing ability of plasma) and oxidative stress markers (TBARS = thiobarbituric acid reactive substances; F2-isoprostanes); for each parameters absolute plasma values are depicted in graphs A, C, E, and G, whereas graphs B, D, F, and H show incremental or decremental values relative to values on d 1: control (■), rutin (□), quercetin aglycone (■), and green tea extract (■); $^{a-b}$LSM with different superscripts differ; values are LSM ± SE, n = 7 per group.

3.3.4 Plasma concentrations of metabolites and hormones

Plasma concentrations of total protein increased ($P < 0.001$) and plasma concentrations of albumin decreased ($P < 0.001$) from d 1 to d 5 in all groups (Table 3.1). After d 5, there was a slight decrease ($P < 0.05$) for total protein and a slight increase for albumin ($P < 0.05$) until the end of the study. Plasma concentrations of urea decreased ($P < 0.01$) until d 12 in all groups and then remained relatively low (Table 3.1). Plasma glucose concentrations slightly increased ($P = 0.01$) in most groups from d 1 to d 5 and then variably changed up to d 26 (Table 3.1). Plasma lactate concentrations sharply decreased after birth up to d 5 but then remained stable up to d 26 (Table 3.1). Plasma NEFA concentrations decreased up to d 5 of life and partly increased again until d 12 (Table 3.1). Plasma insulin and cortisol concentrations decreased after birth in most groups ($P < 0.001$) and then remained low (Table 3.1). All plasma concentrations measured were comparable among groups except for plasma cortisol, where the decrease after birth differed among groups reflected by group × time interaction ($P < 0.001$).

Table 3.2. Blood plasma concentrations of metabolic traits and hormones of calves after no flavonoid (CTRL), quercetin aglycone (QA), rutin (RU) or of a green-tea extract feeding containing mainly catechins (CA) from d 5 to d 26. Samples are taken before morning milk feeding occurred.

Item[1]	Day	Group				SE	ANOVA (*P*-value)		
		CA	CTRL	QA	RU		Group	Day	Group × Day
Total protein, g/L	1	50.6	50.3	50.0	50.3	1.34	0.33	0.00	0.63
	5	60.3	62.7	58.8	61.4				
	12	58.3	60.6	59.4	61.1				
	19	55.6	58.6	58.0	59.0				
	26	56.4	58.0	55.7	58.4				
Albumin, g/L	1	26.1	25.7	24.9	25.2	0.66	0.27	0.00	0.88
	5	24.0	23.7	23.4	23.5				
	12	26.6	24.6	24.4	24.9				
	19	26.0	25.2	25.6	25.4				
	26	26.7	25.6	25.7	25.9				
Urea, mmol/L	1	3.48	3.61	3.84	3.23	0.26	0.74	0.00	0.78
	5	2.61	2.52	2.33	2.31				
	12	2.02	1.40	1.98	1.72				
	19	1.58	1.44	1.55	1.72				
	26	1.43	1.34	1.63	1.52				
Glucose, mmol/L	1	5.46	4.12	4.93	4.98	0.32	0.56	0.00	0.19
	5	5.45	5.31	5.84	5.35				
	12	5.12	4.87	4.94	5.03				
	19	4.76	5.30	5.50	4.71				
	26	5.39	5.26	5.38	5.16				

Table 3.2. Continuation.

Item[1]	Day	Group				SE	ANOVA (*P*-value)		
		CA	CTRL	QA	RU		Group	Day	Group × Day
Lactate, mmol/L	1	5.53	3.02	5.56	4.11	0.77	0.80	0.00	0.50
	5	0.76	0.76	1.88	0.76				
	12	0.59	0.52	0.57	0.62				
	19	0.55	0.43	0.66	0.61				
	26	0.56	0.48	0.49	0.56				
NEFA, µmol/L	1	552	917	674	648	90.8	0.53	0.00	0.28
	5	283	220	248	211				
	12	215	469	344	374				
	19	364	309	295	361				
	26	288	280	276	205				
Insulin, µg/L	1	1.49	0.67	0.65	0.35	0.19	0.23	0.00	0.22
	5	0.44	0.44	0.37	0.32				
	12	0.20	0.25	0.35	0.19				
	19	0.19	0.17	0.17	0.22				
	26	0.16	0.23	0.15	0.15				
Cortisol, ng/mL	1	91.07[a]	55.00[b]	69.77[ab]	88.60[a]	5.42	0.33	0.00	0.00
	5	19.07	19.06	32.06	23.40				
	12	8.10	7.87	11.73	11.96				
	19	6.84	4.60	8.34	10.30				
	26	4.66	4.17	7.60	6.41				

[a-b]LSM in a row with different superscripts differ ($P < 0.05$).
[1] Values are LSM ± SE, n = 7 per group.

3.4 Discussion

At birth BW was not different among groups, as planned, and postnatal growth performance was not affected by treatment. The lack of differences in feed intake and ADG during flavonoid administration is in line with findings of Oliveira et al. (2010) after feeding a flavonoid-rich pomegranate extract to calves for the first 30 d of life. Because concentrate intake did not differ among groups, rumen development during first month of life was unlikely changed by flavonoid administration, as supported by low plasma concentrations of β-hydroxybutyrate that did not increase in flavonoid-treated calves during the experimental period (Maciej and Hammon, unpublished observations). Nevertheless, plant extracts have the potential to affect rumen development, but may be only effective in older calves (Greathead, 2003).

The impact of flavonoids on health status is discussed controversially. Nielsen (2008) found fewer diarrhea in weaned piglets after feeding natural feed additives containing a high amount of flavonoids. In contrast, Oliveira et al. (2010) reported no effects on health parameters, like incidence of diarrhea, by feeding a polyphenol-rich feed to newborn calves. Interestingly, we found an improved fecal score in CA compared to CTRL calves. In recent studies GTE were shown to inhibit diarrhea in newborn calves (Ishihara et al., 2001). In addition, the number of treatments was markedly reduced in the study of Ishihara et al. (2001), confirming our results of numerically less treated calves because of diarrhea in CA.

Plasma antioxidant capacity of calves, based on TEAC and FRAP measurements, was not influenced by flavonoid feeding in this study. However, the sharp changes of both markers from d 1 to d 5 again highlights the role of colostrum feeding (Blum and Hammon, 2000; Hammon et al., 2013). Measurement of TEAC is dependent on the vitamin E analogue trolox. Because vitamin E is provided by colostrum feeding (Blum et al., 1997; Lindmark-Månsson and Åkesson, 2000; Zanker et al., 2000), and plasma TEAC also increased rapidly after first colostrum intake in calves. Our findings on FRAP agree with those of Gaál et al. (2006) that also showed a decline of this parameter after first colostrum intake. Urea is a major factor influencing FRAP (Benzie and Strain, 1996), and albumin and urea are factors influencing TEAC (Miller et al., 1993), but these metabolites only changed in time and were similar among groups. Although plasma metabolites were not affected by treatment, decremental changes of FRAP

values from d 12 to d 19 in CTRL, and from d 5 to d 12 in RU, reflect changes of antioxidative capacity with time.

Oxidative stress markers (TBARS and F2-Isoprostanes) did not follow a clear time-dependent trend as seen for TEAC and FRAP. Nevertheless, TBARS increased in time in CTRL and decreased in CA, suggesting less oxidative stress in calves fed GTE. On the other hand, F2-isoprostane concentrations in plasma decreased only in CTRL, indicating more oxidative stress. In previous studies on calves, plasma concentrations of oxidative stress markers distinctly increased when calves were sick or stressed (Erisir et al., 2013; Ahmed and Hassan, 2007; Al-Qudah, 2009). This suggests that flavonoid feeding to healthy calves, as in the present study, may not change oxidative stress markers. A polyphenol-rich diet in humans (Nieman et al., 2013), lactating cows (Gohlke et al., 2013), pigs (Lühring et al., 2011) or rats (Igarashi and Ohmuma, 1995) also led only to slight or no changes of markers of antioxidant activity or oxidative stress.

In general, methods for determination of TBARS and F2-isoprostanes were discussed to be inappropriate because of too low specificity and concomitantly too high inter-assay variation (Rimbach et al., 1999; Halliwell and Whiteman, 2004; Celi, 2011). Furthermore, antioxidative status tremendously differs between individuals and depends on medical treatment (Farombi, 2001) and season (Bernabucci et al., 2002). In addition, plasma flavonol concentrations were relatively low since blood sampling occurred in the pre-prandial stage and postprandial plasma flavonol concentration in calves decreased rapidly with age (Maciej et al., 2015). Therefore, variable results concerning antioxidative status or oxidative stress markers can be expected. Also, the amount of flavonoid used in this study was based on previous studies in pigs and dogs (Lesser et al., 2004; Reinboth et al., 2010; Lühring et al., 2011), which may not be adequate for colostrum- and milk-fed calves. The determination of other parameters, such as of protein damage, determination of vitamins E and C or of enzymatic antioxidants might be more appropriate.

We assumed that quercetin feeding may affect metabolic status in calves as some authors postulate effects of flavonoids on glucose metabolism (Cermak et al., 2004; Shetty et al., 2004; Gohlke et al, 2013). However, we found no effects on pre-prandial plasma glucose concentrations by flavonoid feeding in this study. The increase of plasma glucose concentrations after birth reflects lactose intake by colostrum and milk

feeding as well as an increasing endogenous glucose production with age (Hammon et al., 2013). Because plasma glucose is highly regulated with the goal to maintain euglycemia, concentrations were relatively stable throughout the study and corresponded to earlier presented data measured in young milk-fed calves (Hadorn et al., 1997; Rauprich et al., 2000; Nussbaum et al., 2002). Plasma concentrations of other metabolites and insulin were also not affected by flavonoid feeding and simply reflected physiological changes during first month of life (Nussbaum et al., 2002; Schiessler et al., 2002; Hammon et al., 2012). Differences in plasma cortisol concentrations among groups on d 1 were independent of flavonoid administration as blood was sampled before flavonoid supplementation.

In conclusion, effects of flavonoid treatment were much less than hoped or expected. However, reduced medical treatment, fewer incidences of diarrhea, and a slight effect on oxidative stress markers may indicate a potential health-promoting effect of catechins in this study. Nevertheless, evidence for biological effects of flavonoid-containing supplements is not available for young calves. Feeding higher doses of flavonoids than in this study may possibly cause more distinct changes of the metabolic or antioxidative status in neonatal calves. In addition, use of more specific markers of anti-oxidative capacity or stress may be helpful in future studies.

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Chapter 4

General Discussion

General Discussion

Rearing neonatal calves healthy and economically is still a major challenge in today's dairy farming. Feed supplements are often claimed to provide an effective and easy way to improve performance of animals, but the actual use of such supplements marketed is manifold. Since flavonoids are ingredients in all higher plants with numerous health-promoting properties like acting antioxidative, antiviral, or anti-inflammatory, and beneficial effects on metabolic key enzymes and even antidiarrheal remedies (Chapter 1; Gálvez et al., 1995; Rao et al., 1997; Middleton et al., 2000), their use in dairy nutrition is comprehensible. However, biological effects *in vivo* require systemic BV. Bioavailability in particular means the absorption, distribution, metabolism, and at least the excretion from the organism. For calves, systemic BV is widely unknown so far. Therefore, this study was performed to examine BV and the biological effects of the most abundant flavonoids quercetin and catechin in neonatal calves.

In the first study of this thesis (Chapter 2) the systemically availability of quercetin, either applied as quercetin aglycone or applied as quercetin glucorhamnoside rutin linked to the sugar rutinose was evaluated in newborn calves on d 2 and on d 29 of life to investigate two different ontogenetic states. In the second study (Chapter 3) possible influences of quercetin as aglycone or as rutin, and furthermore of catechin on the metabolic and antioxidative status in calves were examined during a 3-wk feeding from d 5 to 26 of life.

In both studies of this thesis, quercetin aglycone and rutin were supplemented at an amount of 30 µmol/kg of BW per d. This dosage based results from companion studies in dairy cows (Berger et al., 2012; Gohlke et al., 2013). Both quercetin sources were fed as pure substances, suspended in 10 mL of water to enable oral apply of flavonoids during milk feeding. Therefore, possible effects can be deduced exclusively from flavonoid feeding what would not be the case when feeding plant extracts with mixed compositions or other natural products like propolis, as seen in a number of other studies in calves (Yaghoubi et al., 2008; Oliveira et al., 2010). In contrast to quercetin feeding catechin could not be fed as pure substance, but was fed by a GTE on a level of 10 mg/kg of BW per d of original substance, containing 70.3 % catechins. Dosage of catechins was aligned to quercetin dosage and is shown in Table 1 of the appendix of

this thesis. However, plasma catechin concentrations were below the detection limit (data not shown), thus in further studies higher dosages should be applied.

Studies on BV of flavonoids are conducted for a wide variety of species including humans (Erlund et al., 2000; Egert et al., 2008), pigs (Ader et al., 2000; Lesser et al., 2004; Cermak et al., 2003), rats (Manach et al., 1997, 1999), dogs (Reinboth et al., 2010), horses (Wein and Wolffram, 2013), and cows (Berger et al., 2012; Gohlke et al., 2013). The enormous differences among the species and ages impeded a transfer of findings to neonatal calves (Chapter 1, section 2.2.5). Hence, this thesis shall be a basis for further more specific investigations on flavonoid feeding in calves. Next to differences among species as mentioned above, great inter-individual differences among calves were found in these experiments, confirming previous studies for plasma flavonol concentrations attributed to individual enzyme expression or differences in intestinal bacterial colonization (Németh et al. 2003; Manach et al., 2005). Calves for these studies originated from two distinct neighboring farms, thus genetic background, feeding of dams, and further external influences may partly account for differences found. Such impacts could be avoided by selecting calves from just one dairy operation with a common pedigree.

In the first study of this thesis (Chapter 2), quercetin was systemically available reflected by increased concentrations of total flavonols, quercetin, and the quercetin metabolites tamarixetin, isorhamnetin, and kaempferol in blood plasma of neonatal calves, both after administration of quercetin as aglycone and as glucorhamnoside rutin. Neonatal calves are regarded as functional monogastrics until development of the reticulorumen allows rumination (Drackley, 2008). Therefore, BV in neonatal calves is more comparable to monogastric, but not to ruminant species as quercetin aglycone was better absorbed than rutin. This is in line with a decreasing BV of quercetin with ontogenesis as on d 29 a higher microbial fermentation thus lower BV due to partial ruminal degradation of quercetin was expected (Heinrichs and Jones, 2003; Guilloteau et al., 2009; Berger et al., 2012).

However, absorption of quercetin occurred slowly, reflected in delayed and lower maximal reached plasma concentrations than found in monogastrics (Lesser et al., 2004; Reinboth et al., 2010; Chapter 2) or in adult cattle (Berger et al., 2012; Gohlke et al., 2013; Chapter 2), although applying comparable amounts of quercetin. This once more underpins that results from studies on BV of other species or adult cattle cannot be

deduced to young calves. Furthermore, a delayed absorption of quercetin as glucorhamnoside rutin, but not as its aglycone was seen as result of different absorption sites in monogastrics (Hollman et al., 1997; Manach et al., 1997; Chapter 1). On the contrary, in neonatal calves quercetin aglycone and rutin administration showed the same slow increase in plasma concentrations, indicating similar absorption sites as well as a special feature in milk-fed calves because of abomasal milk clotting (Chapter 2). To exclude interactions with milk fed, flavonoids may be administered with water only instead of milk, which should result in a faster increase of plasma flavonol concentrations. Anyway, to determine the exact place of absorption and metabolism of flavonoids in calves, further studies are required like intraruminal or intraduodenal application of flavonoids or *in vitro* absorption tests of flavonoids with different bovine gastrointestinal tissues.

Aside from this, the slow absorption of flavonols during BV studies (Chapter 2) seems to be associated with the accumulation of quercetin in plasma during long-term administration (Chapter 3). When elimination half-lives of flavonoids are long, repeated absorption of flavonoids can cause accumulation in blood plasma (Aherne and O'Brien, 2002; Chapter 1). Again, calves showed differences to other monogastrics like pigs, where long-term quercetin feeding did not lead to an accumulation in plasma, mainly due to much faster absorption of quercetin (Bieger et al., 2008). In calves, accumulation of flavonols in plasma during long-term administration was especially seen for rutin (Chapter 3) what is in line with findings from BV studies of a delayed peak in plasma flavonol concentrations after rutin feeding, especially on d 2 (Chapter 2).

Interestingly, even without dietary flavonoid supplementation calves showed measurable plasma flavonol concentrations during BV studies (Chapter 2) and during long-term administration (data not shown; Chapter 3). This was particularly seen on day 2 when colostrum was fed (Chapter 2) indicating once more the outstanding role of colostrum for health and welfare of calves (Hammon et al., 2013). In addition, to further clarify the source of flavonols, analysis of the respective composition of colostrum or calf feed is required. In the superordinate context of these studies, bovine milk or colostrum are probable flavonoid sources in human nutrition as well as veal rich in flavonoids as so called functional food. Precondition for this would be further studies examining the distribution of flavonoids in calves and distinct tissue analyses after flavonoid absorption (Chapter 1).

However, concentrations of metabolites and hormones measured postprandial during BV studies (Chapter 2) or basal during long-term flavonoid treatment (Chapter 3) were in line with previous studies on calves without dietary flavonoid supplementation, indicating physiological conditions and an unaffected metabolic status of calves by flavonoid feeding. Results of basal blood samples during long-term flavonoid administration (Chapter 3) confirmed the findings of unaffected metabolic parameters found in BV studies (Chapter 2). Flavonoid administration at higher dosage and a more distinct focus on glucose metabolism during the experimental design putatively may reveal effects as found in companion studies on calves (Gruse et al., 2014) or in monogastric animals showing an inhibited glucose uptake in intestinal cells after quercetin feeding (Cermak et al., 2004). Nevertheless, all data indicated the use of flavonoids in sensitive neonatal calves is harmless. This is noteworthy as secondary plant metabolites in the past mainly counted as anti-nutritive factors (Durmic and Blache, 2012).

Markers of oxidative stress and of the antioxidative capacity in calves during long-term administration of quercetin and catechin were barely influenced by feeding regime, but influenced by time, reflected by physiological changes during maturation (Chapter 3). Colostrum as source for antioxidants like vitamins and enzymes (Lindmark-Månsson and Åkesson, 2000) was assumed to induce at least most of the time effects of the antioxidative status in this study. The distinct increase of TE (unit for TEAC) and the decrease of ASCE (as unit for FRAP) from shortly after birth to d 5 of life demonstrated the important role of feed intake. This was the first study proofing this outstanding influence of feed intake. However, similar changes in plasma concentrations were not seen for TBARS or F2-isoprostanes as oxidative stress markers. Therefore, relative changes from d 1 to other d were evaluated, which pointed out a possible role of catechin feeding. Taken together, reduced medical treatment, better fecal scores and a slight impact on oxidative stress markers possibly indicate health-promoting effects of catechins in calves. This is especially interesting und should be further examined in regard to the low plasma catechin concentrations found.

Nevertheless, in this study changes in oxidative stress markers by flavonoid feeding were weak (Chapter 3) when compared to other studies showing more distinct differences in oxidative stress markers mainly in obviously impaired calves (Wernicki et al., 2006; Ahmed and Hassan, 2007; Al-Qudah, 2009). This was not true in the

studies of this thesis, where all calves were kept under similar conditions. Furthermore, in contrast to plasma metabolites like glucose or protein there is a lack of basic information, like data for physiological ranges, concerning markers for antioxidative capacity and oxidative stress. Anyway, this may be explained by the huge variation found in the distinct studies and the different methods of analysis (Chapter 1, 3), raising the question whether there are more appropriate markers to determine the antioxidative status of calves or if additional parameters should be used. As presented previously (Chapter 1; section 2.3.3) conceivable suggestions are the determination of vitamins E and C, carotenoids, bilirubin or glutathione as well as enzymatic antioxidants like superoxide-dismutase, catalase or glutathione-peroxidase. Moreover, a more closely setting of sample collection over longer time periods would allow a deeper insight in kinetics of flavonoid action. Additionally, in these studies only 7 calves per group were investigated. Using more animals per group would let findings become more conclusive.

On the other hand, dietary flavonoid supplementation, namely catechin feeding, seemed to improve health status reflected in fewer sick calves and less diarrhea, although plasma catechin concentrations were below the detection limit (Chapter 3). However, biological effects not only depended on absolute plasma concentrations, but also on concentrations in target tissues, which is needed to be examined in further studies. A possible approach is comparing directly the effects of flavonoid supplementation from d of birth to a control group without flavonoid supplementation or treating sick calves with flavonoids compared to sick calves without flavonoid treatment with both approaches using an adequate number of animals. Furthermore, the chemical form or the galenical form in general, in which the flavonoid is applied, greatly influences BV and thus the biological effects (Chapter 1) as may be seen in studies that compare quercetin aglycone or rutin administration to natural plant extracts containing quercetin.

In previous studies on BV, urine and feces collection were the appropriate methods used as due to urine and feces analyses, the excretion of the investigated substance can be verified. Since this thesis focused on the more fundamental question if flavonoids were absorbed at all, no excrement was analyzed.

In conclusion, these studies clearly showed for the first time, that quercetin is systemically available in newborn calves. This is a requirement for biological effects in the organism which already are claimed by feed producers in practical dairy nutrition.

The great influence of maturation and ontogenetic stage of young calves on BV of flavonoids was demonstrated just as the influence of the chemical form of the administered flavonoids. It was evaluated to what extend flavonoids affect the metabolic and antioxidative status in neonatal calves, shown in slight changes of the respective markers. This all contributes to a better understanding of the usage, the mode of action, and the benefit of flavonoids as ‘natural’ health-promoting feed supplements.

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Summary

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Flavonoids are secondary plant metabolites with numerous health-promoting properties. The feeding industry widely use flavonoids as ‘green’ feed supplements even scientific evidence of their value are often hypothetical. Due to their high susceptibility to diseases, the use of flavonoids in neonatal calves might be especially useful. However, to determine biological effects *in vivo*, flavonoids must be systemically available in calves, but this is not known yet. Therefore, the aim of this study was to determine BV and their biological effects of the most abundant flavonoids quercetin and catechin in neonatal calves.

In part one of the study, calves (n = 7 per group) were fed quercetin as aglycone or as glucorhamnoside rutin or no flavonoid (control group) on d 2 and 29 of life. Blood samples were taken in defined time intervals before and until 48 h after flavonoid administration via jugular vein catheters. Quercetin and its methylated (isorhamnetin, tamarixetin) and dehydroxylated (kaempferol) metabolites were analyzed in blood plasma using HPLC with subsequent fluorescence detection. It was shown that quercetin is systemically available both as aglycone and as rutin in neonatal calves, but BV of quercetin aglycone was higher than BV of rutin as previously shown in monogastric species. Furthermore, BV of quercetin and its metabolites was greater on d 2 than on d 29 of life and the proportion of quercetin metabolites differed greatly between the two d, which possibly can deduced to maturation of the gastrointestinal tract.

In part two of the study effects of a three-wk administration of quercetin aglycone, rutin or a green tea extract containing mainly catechins, on performance, health, and the antioxidative capacity and oxidative stress in neonatal calves were evaluated. A fourth group was used as control group (n = 7 calves per group) and did not receive any flavonoid sopplementation. No group differences were found for zootechnical parameters, but a slight benefit in health status when catechins were fed. Effects of flavonoid feeding on metabolic status were not seen in this study, but total protein, albumin, urea, lactate, glucose, NEFA, insulin, and cortisol reflected typical time changes during postnatal calf development. Markers of the antioxidative capacity in blood plasma (TEAC, FRAP) showed incisive differences in time, seen in a sharp increase of TEAC and in a decrease of FRAP plasma concentrations postpartum.

However, oxidative stress markers (TBARS, F2-isoprostanes) did not illustrate distinct group and time effects due to great interindividual variances.

Taken together, this study clearly demonstrated that the bioavailability of quercetin is determined by its chemical form and depends to a major extent on physiological processes of digestion and gastrointestinal maturation in calves. In contrast, the influence of flavonoids on the antioxidative status did not show clear results, thus an improvement of the antioxidative status in neonatal calves by flavonoid administration cannot be confirmed by this study.

Zusammenfassung

Flavonoide sind sekundäre Pflanzeninhaltsstoffe, denen zahlreiche gesundheitsfördernde Eigenschaften zugesprochen werden. In der Futtermittelindustrie haben Flavonoide deshalb als „grüne“ Zusatzstoffe bereits weitreichend Einzug erhalten, auch wenn wissenschaftlich fundierte Nachweise bezüglich ihrer Wirkung im Nutztier oftmals hypothetisch sind. Besonders für neugeborene Kälber scheint aufgrund ihrer erhöhten Krankheitsanfälligkeit der Einsatz von pflanzlichen Zusatzstoffen sinnvoll. Allerdings müssten Flavonoide für biologische Effekte systemisch im Kalb verfügbar sein, wofür es bis jetzt noch keine Belege gibt. Somit war es Ziel dieser Arbeit die Bioverfügbarkeit und die biologischen Effekte der weit verbreiteten Flavonoide Quercetin und Catechin beim neugeborenen Kalb zu untersuchen.

Dazu erfolgte im ersten Teil dieser Studie die Fütterung von Quercetin als reines Aglykon oder mit entsprechendem Zuckerrest als Glukorhamnosid Rutin am 2. und 29. Lebenstag der Kälber (n = 7 pro Gruppe). Vor, sowie bis zu 48 Stunden nach der Flavonoidapplikation, wurden in definierten Zeitabständen Blutproben gewonnen und der Gehalt an Quercetin sowie der jeweiligen methylierten (Isorhamnetin, Tamarixetin) und dehydroxylierten (Kaempferol) Metaboliten mittels Hochleistungsflüssigkeitschromatographie mit anschließender Fluoreszenzdetektion analysiert. Es konnte gezeigt werden, dass Quercetin sowohl als Aglykon als auch als Rutin systemisch im Kalb verfügbar war, wobei die systemische Verfügbarkeit von Quercetin nach Fütterung des Aglycons höher war als nach Fütterung von Rutin. Weiterhin wurde am 2. Lebenstag eine deutlich höhere Bioverfügbarkeit von Quercetin und seinen Metaboliten festgestellt als am 29. Lebenstag. Auch war der Anteil der einzelnen Quercetinmetabolite an der Gesamtflavanolkonzentration im Blutplasma stark verändert, was möglicherweise auf den unterschiedlichen Grad der Darmentwicklung am 2. und 29. Lebenstag zurückgeführt werden kann.

Im zweiten Teil der Studie wurde die Leistungsentwicklung, der Gesundheitsstatus sowie die antioxidative Kapazität und der oxidative Stress bei Kälbern untersucht, denen über einen Zeitraum von drei Wochen entweder Quercetin in Form des Aglycons oder als Rutin oder ein Grünteeextrakt, der hauptsächlich Catechine enthielt, verabreicht wurde. Eine vierte Kälbergruppe bekam keine Flavonoide zugefüttert und diente als Kontrollgruppe (n = 7 pro Gruppe). Hierbei konnten in den erhobenen zootechnischen

Parametern keine Gruppenunterschiede, jedoch ein leichter Vorteil durch die Fütterung von Catechin im Gesundheitsstatus, festgestellt werden. Effekte der Flavonoidfütterung auf den Stoffwechsel der Kälber, ausgedrückt durch Gesamtprotein, Albumin, Harnstoff, Laktat, Glukose, nicht-veresterte Fettsäuren, Insulin und Cortisol, konnten in dieser Studie nicht dargelegt werden, jedoch wiesen diese Parameter entwicklungsphysiologische Zeitveränderungen auf. Marker der antioxidativen Kapazität im Blutplasma (TEAC, FRAP) zeigten prägnante Zeitunterschiede, ausgedrückt durch einen deutlichen Anstieg der TEAC und einen ausgeprägten Abfall der FRAP nach der Geburt. Marker für den oxidativen Stress (TBARS, F2-Isoprostane) hingegen zeigten im Blutplasma aufgrund der starken individuellen Streuung keine eindeutigen Behandlungs- und Zeiteffekte.

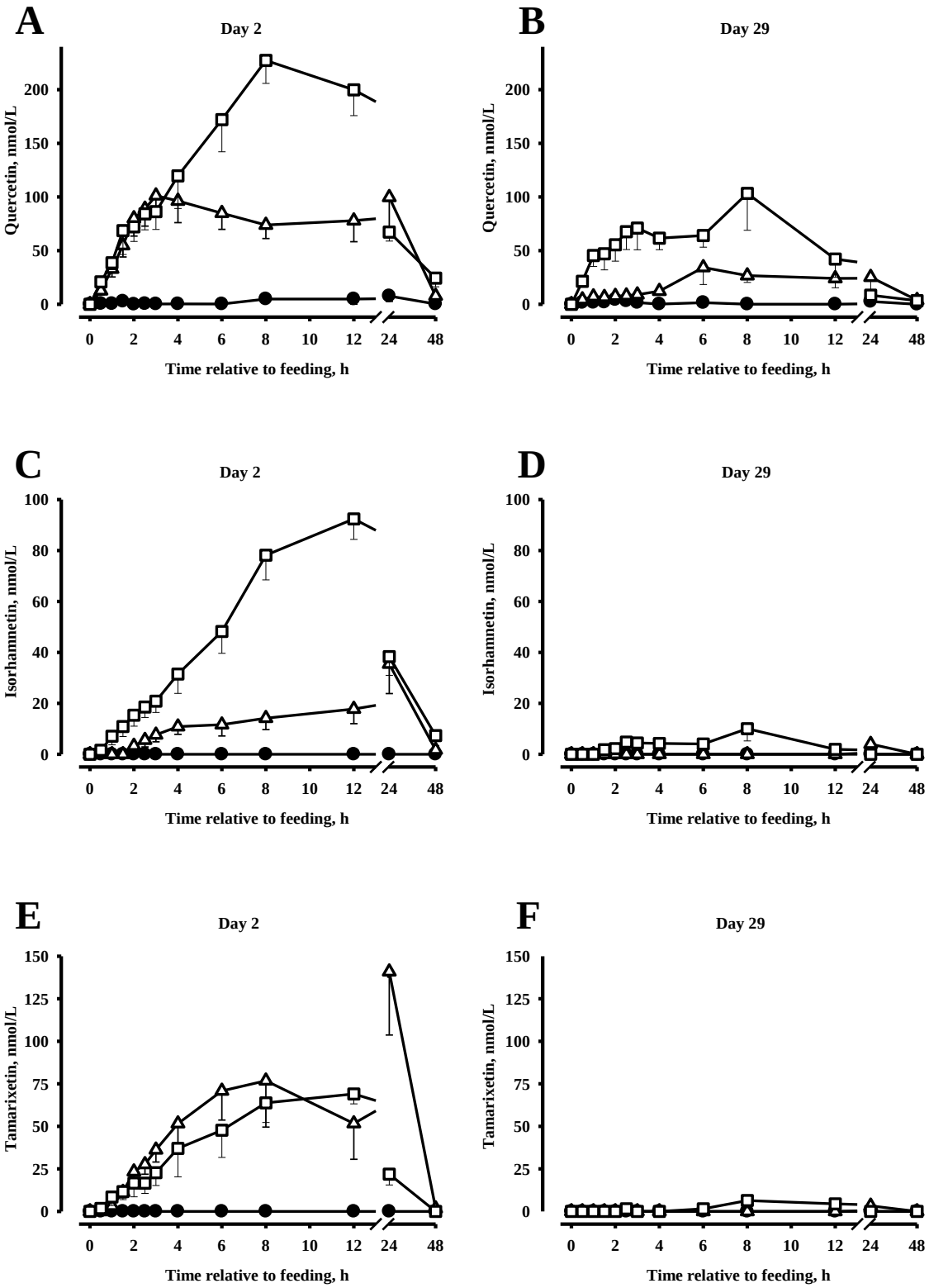
Zusammenfassend konnte mit dieser Studie gezeigt werden, dass die Bioverfügbarkeit von Quercetin durch dessen chemische Form determiniert ist und zum größtenteils von verdauungsphysiologischen Vorgängen und der Darmreifung des Kalbes abhängig ist. Dagegen zeigten die hier durchgeführten Untersuchungen zum Einfluss der Flavonoide auf den antioxidativen Status der Kälber keine eindeutigen Ergebnisse, so dass eine Verbesserung des antioxidativen Status nach Flavonoidgabe bei neugeborenen Kälbern mit dieser Studie nicht bestätigt werden konnte.

Appendix

Table 1 Appendix. Calculation of flavonoid dosage

Item	Formula	Molar mass, g/mol	Flavonoid dosage	
			mg/kg BW	µmol/kg BW
Rutintrihydrate (group RU)	$C_{27}H_{30}O_{16}$ * 3 H_2O	664.6	20	30
Quercetindihydrate (group QA)	$C_{15}H_{10}O_7$ * 2 H_2O	338.3	10	30
Water	H_2O	18.0		
Rutinose	$C_{12}H_{22}O_{16}$	326.3		
Rutin, minus water		610.5		
Rutin, QE[1], minus water and rutinose		284.2/ 302.2[2]	**9**	**30**
Quercetin, QE, minus water		302.2	**9**	**30**
Polyphenon 60 (group CA)			10	
Catechin content 70.3 %			7	
Epigallocatechin	$C_{15}H_{14}O_7$	306.267	1.90	6.20
Epicatechin	$C_{15}H_{14}O_6$	290.260	0.64	2.20
Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	458.370	2.88	6.28
Epicatechin gallate	$C_{22}H_{18}O_{10}$	442.370	0.70	1.58
Gallocatechin gallate	$C_{22}H_{18}O_{11}$	458.370	0.21	0.46
Catechin gallate	$C_{22}H_{18}O_{10}$	442.370	0.03	0.07
Gallocatechin	$C_{15}H_{14}O_7$	306.267	0.52	1.70
Catechin	$C_{15}H_{14}O_6$	290.260	0.14	0.48
Sum, CA			**7**	**19**

[1] QE = Quercetin equivalents.
[2] Only theoretically due to binding of 2 H and 1 O from crystalline water.


A
Day 2
Quercetin, nmol/L
0
50
100
150
200
0 2 4 6 8 10 12 24 48
Time relative to feeding, h
B
Day 29
Quercetin, nmol/L
0
50
100
150
200
0 2 4 6 8 10 12 24 48
Time relative to feeding, h
C
Day 2
Isorhamnetin, nmol/L
0
20
40
60
80
100
0 2 4 6 8 10 12 24 48
Time relative to feeding, h
D
Day 29
Isorhamnetin, nmol/L
0
20
40
60
80
100
0 2 4 6 8 10 12 24 48
Time relative to feeding, h
E
Day 2
Tamarixetin, nmol/L
0
25
50
75
100
125
150
0 2 4 6 8 10 12 24 48
Time relative to feeding, h
F
Day 29
Tamarixetin, nmol/L
0
25
50
75
100
125
150
0 2 4 6 8 10 12 24 48
Time relative to feeding, h

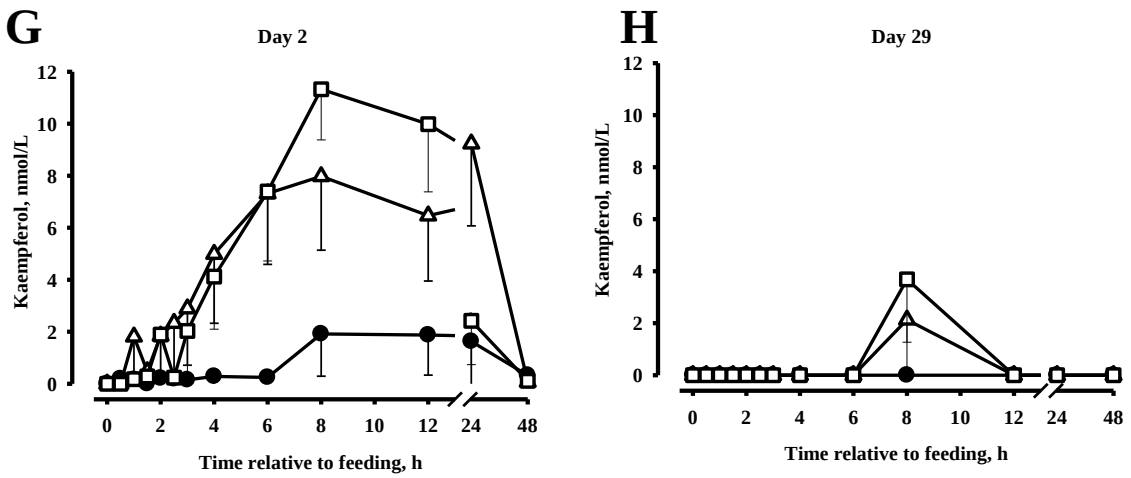
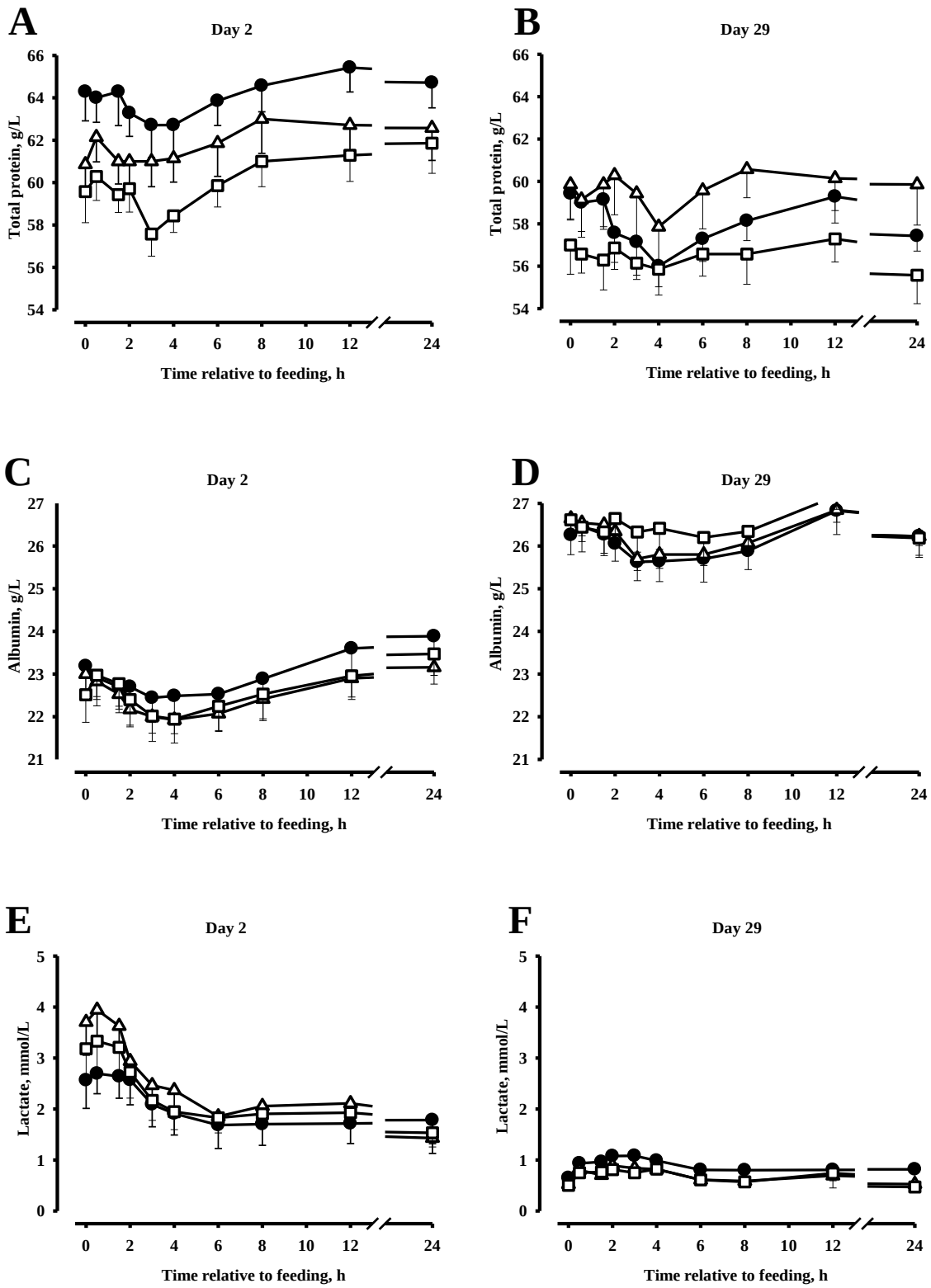


Figure 1 Appendix. Plasma concentration-time curves of quercetin (A, B), isorhamnetin (C, D), tamarixetin (E, F), and kaempferol (G, H) after oral administration of 9 mg of quercetin/kg of BW × d as quercetin aglycone (□), rutin trihydrate (△), or no flavonoid (●) on d 2 and 29 of life. Values are LSM ± SE, n = 7 per group (Chapter 2).


A
Day 2
Total protein, g/L
66
64
62
60
58
56
54
0 2 4 6 8 10 12 24
Time relative to feeding, h
B
Day 29
Total protein, g/L
66
64
62
60
58
56
54
0 2 4 6 8 10 12 24
Time relative to feeding, h
C
Day 2
Albumin, g/L
27
26
25
24
23
22
21
0 2 4 6 8 10 12 24
Time relative to feeding, h
D
Day 29
Albumin, g/L
27
26
25
24
23
22
21
0 2 4 6 8 10 12 24
Time relative to feeding, h
E
Day 2
Lactate, mmol/L
5
4
3
2
1
0
0 2 4 6 8 10 12 24
Time relative to feeding, h
F
Day 29
Lactate, mmol/L
5
4
3
2
1
0
0 2 4 6 8 10 12 24
Time relative to feeding, h

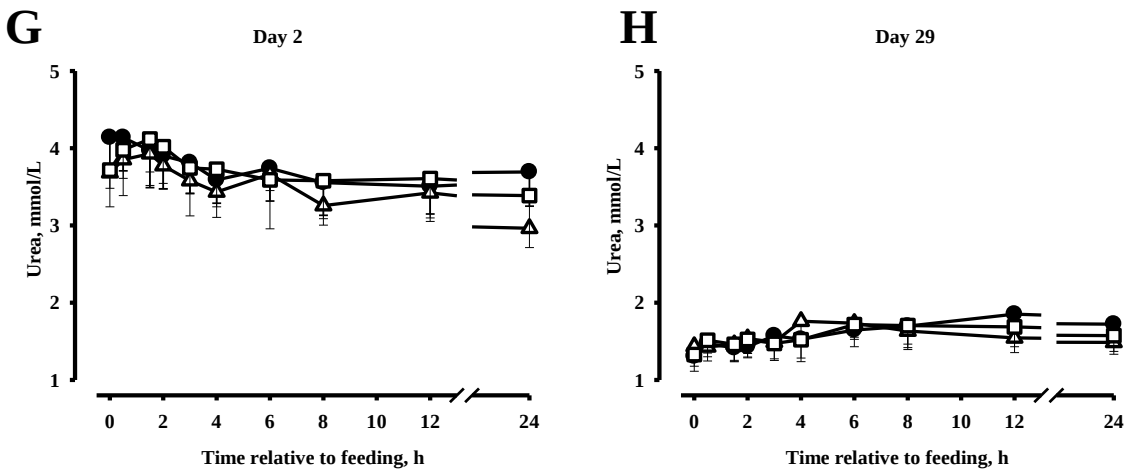
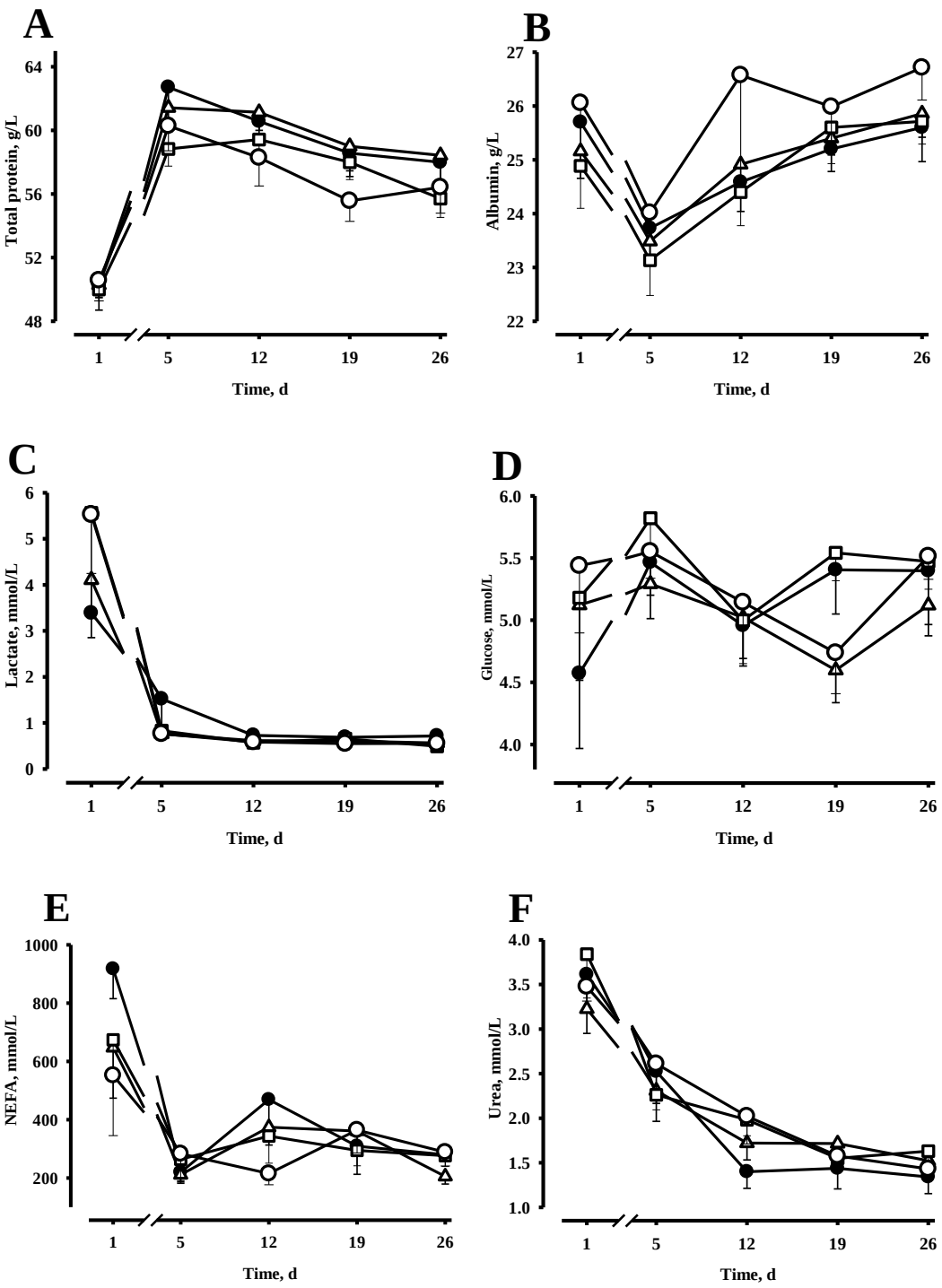


Figure 2 Appendix. Plasma concentrations of total protein (A, B), albumin (C, D), lactate (E, F), and urea (G, H) after oral administration of 9 mg of quercetin/kg of BW × d as quercetin aglycone (☐), rutin trihydrate (△), or no flavonoid (●) on d 2 and 29 of life. Values are LSM ± SE, n = 7 per group (Chapter 2).


A
Total protein, g/L
64
60
56
52
48
1
5
12
19
26
Time, d
B
Albumin, g/L
27
26
25
24
23
22
1
5
12
19
26
Time, d
C
Lactate, mmol/L
6
5
4
3
2
1
0
1
5
12
19
26
Time, d
D
Glucose, mmol/L
6.0
5.5
5.0
4.5
4.0
1
5
12
19
26
Time, d
E
NEFA, mmol/L
1000
800
600
400
200
1
5
12
19
26
Time, d
F
Urea, mmol/L
4.0
3.5
3.0
2.5
2.0
1.5
1.0
1
5
12
19
26
Time, d

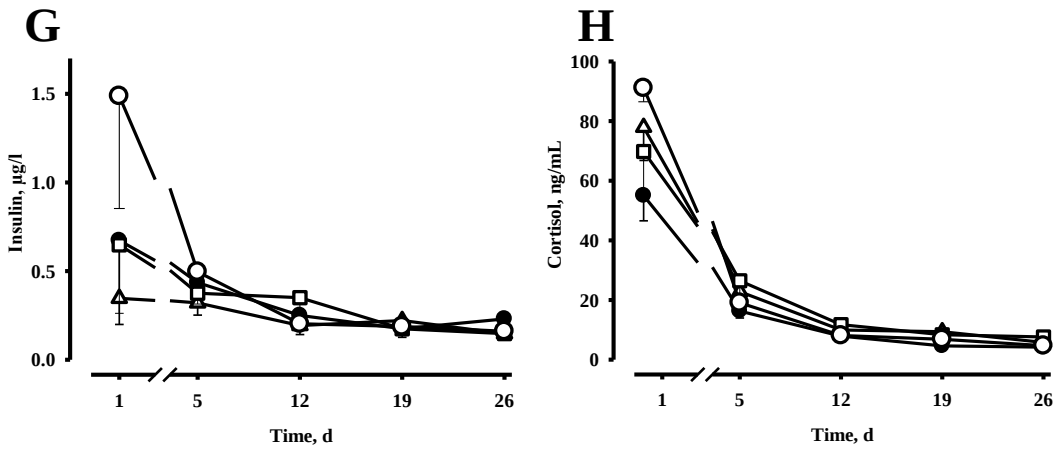


Figure 3 Appendix. Plasma concentrations of total protein (A), albumin (B), lactate (C), glucose (D), NEFA (E), urea (F), insulin (G), and cortisol (H) after oral administration of quercetin aglycone (□), rutin trihydrate (△), a green tea extract (○) or no flavonoid (●). Values are LSM ± SE, n = 7 per group (Chapter 3).

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für die bedingungslose Unterstützung, Liebe, Zuversicht!!!

Krasse Herde…

Curriculum Vitae

Josefine Maciej (née Flor) born on October 02, 1984 in Kühlungsborn

Nationality: German **Civil Status:** Married, 2 children

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Since 10/2011	Doctoral student Agricultural Sciences, Christian Albrechts University Kiel, Faculty of Agricultural and Nutritional Sciences
10/2007 – 02/2010	Master of Science Agriculture Ecology, University of Rostock, Master program: Livestock Sciences, Master Thesis: The use of computer tomography for the evaluation of stomach development in milk-fed calves (Grade 1.0)
10/2004 – 09/2007	Bachelor of Science Agriculture Ecology, University of Rostock
06/2004	Abitur, Gymnasium Sanitz

Work/Professional Experience

06/2010 – 06/2014	Scientific assistant/doctoral student, Leibniz Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology (‘Oskar-Kellner’), Dummerstorf
05/2009 – 07/2009	Student research assistant, University of Göttingen, Research Unit Agriculture and Environment
09/2008 – 02/2009	Agricultural journey through the USA; Internship at the weekly dairy farm family magazine ‘Farmshine’, Brownstown, PA
02/2008 – 04/2008	Internship Landwirtschaftsberatung Mecklenburg-Vorpommern, Schleswig-Holstein (LMS), Bad Doberan
11/2004 – 12/2005	Student research assistant, University of Rostock, Faculty of Agricultural and Environmental Sciences, Institute of Animal Nutrition
08/2004 – 01/2008	Internship/Practical work Landwirtschaftsgesellschaft Prisannewitz

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Since 05/2010	Member of volunteer fire department, Kavelstorf

Kiel; May 04, 2015