Research Article

Gastrointestinal absorption and metabolism of soy isoflavonoids in ileal-canulated swine

Kelly R. Walsh[1], Sara J. Haak[2], Nathan D. Fastinger[1,3], Torsten Bohn[4], Qingguo Tian[4], Donald C. Mahan[1,3], Steven J. Schwartz[1,4] and Mark L. Failla[1,2]

[1] The Ohio State University Interdisciplinary PhD Program in Nutrition, Columbus, OH, USA
[2] The Ohio State University Department of Human Nutrition, Columbus, OH, USA
[3] The Ohio State University Department of Animal Science, Columbus, OH, USA
[4] The Ohio State University Department of Food Science & Technology, Columbus, OH, USA

The relative contribution of the small intestine to absorption and microbial metabolism of ingested isoflavonoids (IFN) was investigated in swine with canulae in distal ilea to facilitate collection of chyme (canula open). Weaned swine were fed a single meal containing ground roasted soybean and corn with canulae open followed by a second test soy diet at 48 h with canulae closed to allow passage of chyme into the large intestine. All remaining feedings were soy-free (corn – casein diet). Ileal effluent and urine were collected for 16 and 48 h, respectively, and analyzed for IFN and microbial metabolites of IFN. IFN in ileal effluent were present entirely as aglycones. IFN equivalents excreted for 24 h after ingesting the soy diet were not significantly different when canulae were open or closed. Urinary IFN aglycone equivalents on day 2 were similar to those on day 1 when canulae remained closed, but less than 10% of that on day 1 when canulae were open for 16 h postfeeding. Urinary concentrations of dihydrodaidzein, dihydrogenistein, *O*-desmethylangolensin, and equol exceeded IFN aglycone equivalents. These findings suggest extensive preabsorptive conversion of IFN glucosides to aglycones in the small intestine and relatively efficient microbial metabolism of IFN in weaned swine.

Keywords: Bioavailability / Isoflavonoids / Metabolism / Soy / Swine

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1 Introduction

Isoflavonoids (IFN) are phytoestrogenic compounds present primarily as glucoside conjugates in nonfermented soy foods [1, 2]. Although numerous studies have addressed the health-promoting properties of IFN, details about their site(s) of absorption are limited. It is well established that IFN glucosides must be deglycosylated to aglycones prior to absorption [3]. Plasma concentrations of IFN rise sharply in humans within 2 h of ingesting soy foods and are generally followed by a later peak at 8 – 12 h [4 – 7]. The latter increase in plasma concentration of IFN suggests a role for the large intestinal microflora in the deglycosylation of IFN glucosides and the absorption of aglycones [3, 8], as well as enterohepatic recirculation of absorbed IFN [9 – 11].

We previously reported that IFN glucosides are stable during simulated oral, gastric, and small intestinal digestion of soy food [12], suggesting that mammalian or microbial enzymes within the gut hydrolyze IFN glucosides. The role of the small intestine in IFN glucoside hydrolysis has been the subject of several reports. Andlauer *et al*. [13, 14] demonstrated that the small intestine deglycosylated IFN glucosides during luminal perfusion in rats. Moreover, daidzein and genistein were excreted in the urine of germ-free rats following consumption of a meal containing IFN glucosides, suggesting that microbial activity is not essential for intestinal deglycosylation of glucosides in this species [15]. Indeed, lactase phlorizin hydrolase from humans, sheep, and rats catalyzes the deglycosylation of IFN [16 – 18].

IFN aglycones can be further metabolized by gut microflora. Some microbial species reduce the aglycone daidzein to dihydrodaidzein (DHD), which can be converted to *O*-desmethylangolensin (*O*-Dma) or equol. Likewise, the

Correspondence: Dr. Mark L. Failla, Department of Human Nutrition, The Ohio State University, 1787 Neil Avenue, 325 Campbell Hall, Columbus, OH 43210, USA
E-mail: failla.3@osu.edu
Fax: +614-292-8880

Abbreviations: DHD, dihydrodaidzein; **DHG,** dihydrogenistein; **GI,** gastrointestinal; **IFN,** isoflavonoid(s); **MDL,** minimum level of detection; ***O*-Dma,** *O*-desmethylangolensin

aglycone genistein can be metabolized to dihydrogenistein (DHG) and 6′-hydroxy-*O*-Dma [19, 20]. Simons *et al*. [21] have reported that fecal microorganisms are capable of converting glycitein to dihydroglycitein which may be metabolized to 5′-methoxy-*O*-Dma and dihydro-6,7,4′-trihydroxyisoflavone.

In the present study, we investigated the gastrointestinal metabolism and absorption of IFN in swine with canulae surgically implanted at the terminal ileum. Swine were selected as a model because of the anatomical and physiological similarities of their digestive system and vasculature with those in humans [22]. The intestinal mucosa from humans and swine also exhibit similar permeabilities to markers of paracellular transport [23]. Metabolism of IFN in the upper gut was determined by analyzing ileal digesta collected from open ileal canulae after feeding a meal containing ground roasted soybeans by HPLC for parent IFN, and HPLC coupled to MS/MS for microbial metabolites of IFN. IFN absorption was estimated by comparing the quantities of deconjugated IFN aglycones and metabolites in urine from each pig when ileal canulae were open (*i.e*., digesta collected) and closed (*i.e*., digesta allowed to enter the large intestine) after feeding the test diet.

2 Materials and methods

2.1 Supplies

Daidzein, genistein, glycitein, and their β-, acetyl-, and malonyl-conjugates were purchased from LC Laboratories (Woburn, MA). The internal standard 2′,4′-dihydroxy-2-phenylacetophenone was obtained from Indofine Chemical Company (Hillsborough, NJ). β-Glucuronidase from *Helix pomatia* (G-1512) and the internal standard flavone (F-2003) were purchased from Sigma Chemical (St. Louis, MO). C-18 SPE columns (#205462) and nylon syringe filters (0.2 and 0.45 μm) were purchased from Alltech Associates (Deerfield, IL). Advantasoy™ Clear IFN extract was provided by Cargill Health Food and Technologies (Wayzata, MN). Commercial zinc oxide ointment (Pfizer, Morris Plains, NJ) was procured from a local pharmacy. Reagents for HPLC and MS analyses were purchased from Fisher Scientific (Fairlawn, NJ) and were of HPLC grade.

2.2 Animals

Crossbred barrows ($N = 6$) weighing 40–50 kg were each surgically implanted with a T-canula at the distal ileum as previously described [24, 25]. Placement of the canula facilitates collection of chyme at the terminal ileum. Chyme that is normally transferred to the large intestine is shunted to the collection bag when the canula is open. Thus, postprandial effluent represents chyme that has undergone oral, gastric, and small intestinal digestion. When the canula is closed, chyme transits from the ileum to the large intestine.

Table 1. Composition of swine diets

Component	Corn–casein (control) (% mass)	Corn–soy (test) (% mass)
Corn	90.75	61.35
Roasted soybeans	–	35.80
Casein	7.20	–
Chromic oxide	–	0.40
Salt	0.20	0.30
Dicalcium phosphate	0.70	1.10
Limestone	0.65	0.55
Trace mineral mix	0.10	0.10
Vitamin premix	0.25	0.25
Se premix	0.15	0.15

The animals were housed in individual stainless steel metabolic crates (0.5 m × 1.5 m) to control food and water intake, facilitate collection of urine and ileal effluent, and prevent the animals from tampering with their canulae. Swine and their crates were washed with soap and water each morning. A commercial zinc-oxide ointment (Pfizer) was applied liberally to canula sites to minimize the potential for infection. Animals were acclimated to their cages for 7 days and fed a standard corn–soy diet formulated to meet or exceed NRC [26] nutrient recommendations before initiating feeding studies. Swine were allowed free access to water throughout the study.

2.3 Diets and experimental design

All diets were prepared at The Ohio State University feed mill (Wooster, OH). A soy-free, corn–casein control diet was fed during washout periods (Table 1). Ground roasted soybean was the source of IFN in the test diet (Table 1). Chromium oxide (0.4%) was added to the test diet as a nonabsorbable marker for recovery of digesta from open ileal canulae. Two separate feeding studies were conducted using different sets of animals. The first study was a qualitative characterization of the kinetics of small intestinal clearance of the test diet and IFN excretion in the urine. The second study quantitatively addressed recovery of IFN and their microbial metabolites in urine and ileal effluent. Data from the first and second studies were not pooled due to differences in the size of animals and the duration of ileal effluent collection.

In the first study, pigs ($N = 6$, approximately 50 kg) were fed the soy-free, corn–casein diet at 0600 and 1800 h for 5 days prior to feeding the test soy diet. Following washout, animals were fed the test diet at 0600 h and ileal effluent was collected and pooled every 2 h until 12 h postingestion. Collection bags were changed at approximately 10 min intervals and contents for each 2 h period were immediately pooled for individual pigs and stored on ice. Once the 2 h collection period was completed, pooled digesta samples were stored at −20°C until analysis. Approximately 85% of

Table 2. Experimental design (study number two)

	Canula position				
	Diets		Open	Closed	Urine
Day	0600	1800	(h)	(h)	Collected
−1	Control[a)]	Control	–	24	+
+1	Test[b)]	Control	16	8	+
+2	Control	Control	–	24	+
+3	Test	Control	–	24	+
+4	Control	Control	–	24	+

a) Corn–casein.
b) Corn–soy diet.

dietary chromium oxide in the test diet was recovered in ileal effluent during the 12 h collection period.

Urine collection was initiated 24 h prior to feeding the test diet (day −1) at 12 h intervals and continued through day +4. Urine was filtered through glass wool to prevent particulate matter from entering collection vessels containing L-ascorbic acid (1.14 mmol) and boric acid (4.85 mmol) as preservatives [27]. The collection vessels were replaced every 12 h and urine volumes were measured. Aliquots of the two 12 h urine pools were combined proportionately into 24 h pools and stored at −20 C until analysis.

The general experimental design for the second study is outlined in Table 2. Pigs ($N = 6$, approximately 40 kg) were fed the control soy-free, corn–casein diet (550 g) at 0600 and 1800 h for 5 days (day −5 to day −1) before feeding the first feeding of the test soy diet. Prior to feeding the test diet, one animal removed its canula and was euthanized. A second animal developed a stomal blockage 8 h after feeding the initial test diet and was eliminated from the study. The remaining four animals completed the study. The first of two soy diets (550 g) was fed at 0600 h on day +1 and ileal canulae remained open until 2200 h to collect digesta and minimize its transfer to the large intestine. These pigs were then fed the control diet (550 g) at 1800 h on day +1 and for both meals on day 2. The test soy diet was fed again at 0600 h on day 3 with ileal canulae closed to allow direct passage of digesta from small intestine to the large intestine. The control diet was fed for the remainder of the study. Urine was collected throughout the study as described above for the first study.

2.4 Incubation of IFN glucosides with ileal effluent

To examine the possibility of *ex vivo* microbial hydrolysis of IFN glucosides in ileal chyme in collection bags, stomal effluent was collected from pigs ($N = 4$) fed the corn–casein control diet and mixed (5:1 v/v) with an aqueous solution of IFN glucosides (100 mg/mL, Advantasoy Clear IFN glucoside-rich extract in water). Aliquots (5 mL) of the effluent/IFN mixture were transferred to petri dishes, covered, and incubated in an anaerobic chamber (Thermo Forma Scientific, Marietta, OH) at either 37°C for 0, 20, and 40 min or at 0°C for 20 min. Samples were stored at −20°C until analysis.

2.5 Extraction of IFN from diets and ileal effluent

Aliquots of the diets were ground to a fine powder with a mortar and pestle prior to IFN extraction. Ileal effluent was thawed, weighed, and homogenized with a blender (Osterizer Galaxie, Oster, Milwaukee, WI). Aliquots were freeze-dried to complete dryness, finely ground, passed through a 0.5 mm mesh screen, and stored at −20°C until analysis. IFN in swine diets and ileal effluent were extracted into acidified ACN as described previously [12, 28]. Extracted samples were centrifuged (Fisher Centrific Centrifuge, Fisher Scientific) at $450 \times g$ for 30 min at room temperature. The solvent was evaporated from aliquots (1 mL) of the supernatants, residues were resolubilized in methanol (1 mL), and filtered (0.2 μm pore size) prior to HPLC analysis.

2.6 Extraction of IFN and their derivatives from swine urine

IFN and their metabolites were extracted from swine urine as previously described with slight modifications [29, 30]. Aliquots of urine samples collected during the 24 h period before feeding the first test diet and 0–24 and 24–48 h after each test diet were separately analyzed to measure IFN equivalents excreted daily by each pig. Microbial metabolites of IFN were extracted from pooled urine collected from 0 to 48 h. Aliquots of pooled urine (8 mL) were centrifuged (Avanti J-25, Beckman Coulter, Palo Alto, CA) at $10\,000 \times g$ for 10 min at 4°C. Sodium acetate buffer (1.5 mL, 0.2 mol/L, pH 4.0) and internal standard (25 μmol/L 2′,4′-dihydroxy-2-phenylacetophenone) were added to filtered (0.45 μm) aliquots (5 mL) of supernatants. Urine samples were drawn through C-18 SPE columns using a vacuum manifold (Alltech Associates #210351) after sequential conditioning with methanol (6 mL) and sodium acetate buffer (6 mL, 0.2 mol/L, pH 4.0). Columns were rinsed with sodium acetate buffer (4 mL, 0.2 mol/L, pH 4.0), water (1 mL), and methanol (1 mL), respectively. Methanol (7 mL) was drawn through the columns to elute IFN and their derivatives into collection vials. Samples were heated to 37°C in a water bath and the solvent was evaporated under a stream of nitrogen. Residues were dissolved in 1 mL sodium acetate buffer (0.2 mol/L, pH 5.0) containing 200 units β-glucuronidase. Enzyme-treated samples were incubated in a shaking water bath (Versa-Bath S Model 224, Fisher Scientific) at 50 rpm for 18–22 h at 37°C. IFN and their derivatives were extracted twice into diethyl ether (3.5 mL) and the combined organic phases

were dried under a stream of nitrogen at room temperature. Residues were dissolved in 0.6 mL methanol/water (80:20 v/v) and filtered (0.2 μm pore diameter) before injection into the HPLC system.

2.7 HPLC analysis of IFN

Samples were analyzed for IFN content with a Waters 2695 HPLC (Milford, MA) and a Waters 2996 photodiode array detector (PDA) set to collect data from 210 to 400 nm. The stationary phase consisted of a Waters Nova-Pak C-18 RP column (150 mm × 3.9 mm id., 4 μm, 60 Å pore size) and a Waters Nova-Pak C-18 guard column (20 mm × 3.9 mm id, 4 μm). Different HPLC methodologies were employed to separate IFN from urine samples and diet/effluent samples due to differences in IFN chemical speciation between these matrices. HPLC methodologies for separation of IFN from these matrices have been described in detail elsewhere [12, 30]. Briefly, a mobile phase of 1.0% acetic acid in water v/v (solvent A) and ACN (solvent B) at a flow rate of 0.6 mL/min (25 ± 5°C) was used to separate IFN in extracted diet and ileal effluent samples. The injection volume was 10 μL and the following linear solvent gradient was used: 0–5 min, 85% A; 5–36 min, 85–71% A; 36–44 min, 71–65% A; 44–45 min, 65–15% A; and, 45–50 min, 15–85% A. A mobile phase of 1.0% acetic acid in water v/v (solvent A), ACN (solvent B), and methanol (solvent C) at a flow rate of 0.55 mL/min (25 ± 5°C) was used to separate IFN in extracted urine samples. The injection volume was 10 μL and the following linear solvent gradient was used: 0–1 min, 75% A, 12% B; 1–14 min, 75–49% A, 12–25% B; 14–15 min, 49–10% A, 25–45% B; 15–19 min, no change; 19–20 min, 10–75% A, 45–12% B; and, 20–25 min, no change. Elution profiles and UV absorption spectra of pure IFN standards were used to identify each IFN species.

2.8 HPLC-MS/MS analysis of microbial metabolites of IFN

HPLC-MS/MS was used for the identification and quantification of equol, DHD, DHG, *O*-Dma, and 6-OH-*O*-Dma in ileal effluent and urine. The same extraction procedures and operating parameters used for urine analyses by HPLC were implemented for HPLC-MS/MS analyses. A triple quadrupole mass spectrometer (Micromass, UK) using positive ion ESI and selected reaction monitoring was used to detect DHD (*m*/*z* 257 > 123), DHG (*m*/*z* 273 > 123), *O*-Dma (*m*/*z* 259 > 121), 6-OH-*O*-DMA (*m*/*z* 275 > 121), and equol (*m*/*z* 243 > 133) using their respective calibration curves generated from standards. 2′,4′-dihydroxy-2-phenylacetophenone (*m*/*z* 229 > 137) was used as internal standard during analysis. These transitions were determined using the protonated molecule and the most abundant fragment ion established during analysis of their respective standards. The following system conditions were maintained for analyses: cone voltage of 35 V; capillary voltage, 3.0 kV; source temperature, 120°C; radio frequency lens 1, 50 V; desolvation gas temperature, 500°C at a flow of 16.3 L/min; and, collision energy, 25 eV. Due to limited availability of frozen aliquots of urine, IFN metabolites were not quantified in urine from pigs when ileal canulae were open. The minimum level of detection (MDL) and lower limit of quantification (LLOQ) were determined according to the US Environmental Protection Agency approach [31]. The MDLs determined were 0.003 and 0.072 for equol, 0.002 and 0.048 for *O*-DMA and 6-OH-*O*-DMA, and 0.001 and 0.024 for DHD and DHG, in nanomole *per* milliliter urine and nanomolo *per* gram of dried digesta, respectively. The LLOQ was set three times the MDL.

2.9 Quantitation of chromium oxide

Chromium oxide content of the test diet and ileal chyme samples was determined by neutron activation analysis at the University of Missouri Experiment Station Chemical Laboratories at the University of Missouri (Columbia, MO).

2.10 Statistical analysis

Data for study 1 ($N = 6$) and study 2 ($N = 4$) are expressed as means ± SEM when appropriate. A general linear univariate model was developed to examine the effect of canula status (open or closed) on urinary excretion of IFN. This model incorporated the type of IFN (daidzein, glycitein, genistein, and total), pooled urine collection time (0–24 and 24–48 h), canula status (open or closed), and subject as fixed factors with urinary IFN as the dependent variable. Normal distribution of data was verified using normality plots and Kolmogorov Smirnoff tests. Equality of distribution was verified using box-plots and Levene's test. Urinary IFN metabolites were studied using a paired linear mixed model design with the amount of urinary IFN metabolite as the dependent variable, and the type of IFN metabolite and animal number as fixed factors. Following this test, LSD and Bonferroni posthoc comparisons of statistical significance were performed for individual pairwise comparisons of different IFN metabolites. Differences were considered statistically significant for $p < 0.05$.

3 Results

The majority of the IFN in the test diet were present as glucosides with components of the daidzein, glycitein, and genistein families contributing 375.2 ± 19.4, 90.9 ± 7.2, and 317.3 ± 4.0 μmol, respectively (Table 3). β-, acetyl-, and malonyl-glucosides accounted for 27.0 ± 1.1, 24.9 ± 0.5, and 42.1 ± 1.5% of the total IFN, respectively, and agly-

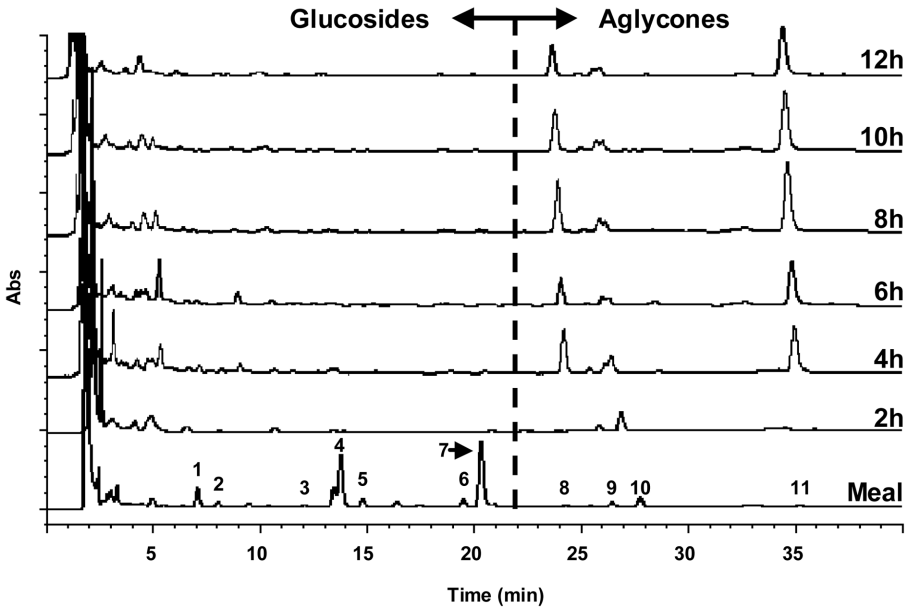

Figure 1. Representative RP HPLC chromatograms of the corn–soy test diet and ileostomal effluent (study number one). Swine ($N = 6$) with ileal canulae were fed a corn–soy test diet to determine the kinetics of small intestinal clearance of the soy test diet. Ileal effluent was collected and pooled for each 2 h interval from 0 to 12 h. Peak identification: (1) daidzin, (2) glycitin, (3) genistin, (4) malonyldaidzin, (5) malonylglycitin, (6) acetyldaidzin, (7) malonylgenistin, (8) daidzein, (9) glycitein, (10) acetylgenistin, and (11) genistein. The corn–soy test diet contained predominantly IFN glucosides, whereas ileal effluent only contained IFN aglycones at all times.

Table 3. IFN content of the corn–soy test diet[a)]

Species	Daidzein (μmol/diet)	Glycitein (μmol/diet)	Genistein (μmol/diet)
Aglycone	23.8 ± 5.9	nd[b)]	23.4 ± 0.4
β-Glucoside	121.0 ± 4.7	42.6 ± 3.1	48.1 ± 0.7
Acetylglucoside	87.2 ± 1.3	18.9 ± 0.7	88.6 ± 1.8
Malonylglucoside	143.3 ± 7.5	29.4 ± 3.4	157.2 ± 1.1
Total glucosides	736.3 ± 24.3 (94.0 ± 3.1%)		
Total aglycones	47.1 ± 6.3 (6.0 ± 0.8%)		

a) Data ($N = 3$) are expressed as means ± SEM of IFN in 550 g of the soy test diet.
b) nd: Not detected.

cones accounted for only 6% of total IFN equivalents. IFN were not detected in the control diet (data not shown).

The first study addressing the kinetics of small intestinal clearance of the soy diet revealed that ingested IFN glucosides were hydrolyzed to aglycones prior to reaching the terminal ileum. IFN aglycones were detected in ileal effluent at all intervals between 2 and 12 h after ingesting the diet with soy, and the majority reached the terminal ileum between 4 and 12 h (Fig. 1). In order to examine the extent to which microflora may have hydrolyzed the glucosides *ex vivo*, ileal effluent collected after swine were fed a corn–casein diet was spiked with a mixture of IFN glucosides and incubated anaerobically. Recoveries of IFN glucosides from the effluent after 20 min at 0°C or 40 min at 37°C were 106 ± 1.2 and 94.5 ± 4.4%, respectively. This observation suggests that *ex vivo* deglycosylation of ingested IFN aglycones was unlikely as bags collecting ileal digesta were changed and placed on ice at approximately 10 min intervals.

Results from the second study followed the same pattern as those from the first study in which IFN glucosides from the test diet were hydrolyzed to aglycones prior to reaching the ileal canula (Fig. 2). The IFN content of digesta collected when canulae were open (0–16 h after meal) was 163.1 ± 51.5 μmol (range: 71.1–272.8 μmol) which represents 20.8 ± 6.6% of the test diet. Daidzein was more abundant in the effluent (91.1 ± 27.9 μmol) than genistein (30.8 ± 10.8 μmol) and glycitein (23.1 ± 6.6 μmol). Although the major microbial metabolites of ingested IFN (*i.e.*, DHD, DHG, and equol) were detected in ileal effluent from all swine (Table 4), the total amounts of daidzein and genistein in the effluent were approximately 140 and 190 times greater than their respective microbial metabolites.

Urine samples were first incubated with β-glucuronidase to convert phase II conjugates of IFN to free IFN aglycones. The enzyme preparation from *H. pomatia* catalyzes hydrolysis of both glucuronide and sulfate conjugates. Daidzein, glycitein, and genistein were not detected in enzyme-treated urine prior to feeding the test soy diet (data not shown). During the 24 h collection period after pigs were fed the test soy diet, total mean urinary IFN equivalents were slightly, but not significantly, lower when ileal canulae were open (20.4 ± 2.8 μmol) compared to canulae closed

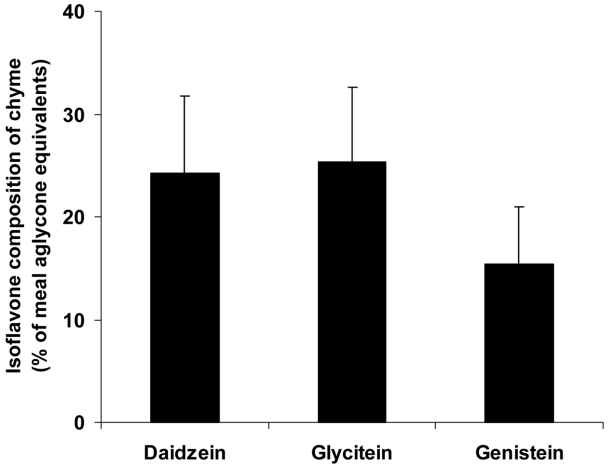

Figure 2. Dietary IFN glucosides are converted to aglycones prior to reaching the terminal ileum in swine with ileal canulae (study number two). Swine were fed the corn–soy test diet and ileostomal effluent was collected for 16 h. The test diet contained primarily IFN glucosides (94.0 ± 3.1%, Table 3), whereas only IFN aglycones were detected in chyme. Data are means ± SEM ($N = 4$) and expressed as percentage of each aglycone in chyme with respect to daidzein, glycitein, or genistein equivalents in the corn–soy test diet.

Table 4. IFN metabolites in ileal effluent of swine following consumption of the corn-soy test diet (study number two)

Metabolites[a)]	μmol ± SEM	Range, μmol	Producers[b)]
DHD	0.30 ± 0.2	0.06–0.94	4/4
DHG	0.26 ± 0.2	0.02–0.88	4/4
O-Dma	0.0	nd[c)]	0/4
6-OH-*O*-Dma	0.003	nd–0.003	1/4
Equol	0.36 ± 0.2	0.17–0.81	4/4

a) Data for ileal effluent ($N = 4$) are means ± SEM where applicable, and represent total quantity for the 16 h collection period. Differences in the amount of metabolite in distal ileal effluent between means were not statistically significant as determined by one-way analysis of variance (ANOVA) followed by Bonferroni posthoc test.
b) Number of animals producing detectable amounts of indicated metabolites.
c) nd: Not detected.

(24.4 ± 9.5 μmol) (Fig. 3a). Urinary excretion of daidzein equivalents was greater than genistein equivalents which exceeded glycitein equivalents during the 24 h period after ingestion of soy test diets regardless of ileal canula position. The quantities of the individual aglycone equivalents excreted in urine were not significantly different when ileal canulae were opened or closed after feeding the test diet (Fig. 3a). Recovery of ingested aglycone equivalents in 24 h urine pools also was not significantly different when canulae were opened *versus* closed (daidzein, 2.6 ± 0.3% *vs*. 3.1 ± 1.3%; glycitein, 3.6 ± 0.9% *vs*. 3.9 ± 1.5%; and genistein, 2.4 ± 0.3% *vs*. 2.9 ± 1.1%, respectively). There was no significant correlation between the amounts of daidzein ($r^2 = 0.33$), glycitein ($r^2 = 0.02$), or genistein ($r^2 = 0.17$) in urine and ileal effluent. Results from the first study revealed no significant differences in the quantities of total and individual aglycone equivalents recovered in 24 h urine samples for pigs when ileal canulae were open *versus* closed after ingestion of the test soy diet. Data between studies were not pooled due to different weights of animals and period of time canulae remained open (*i.e.*, 12 *vs*. 16 h for studies one and two, respectively).

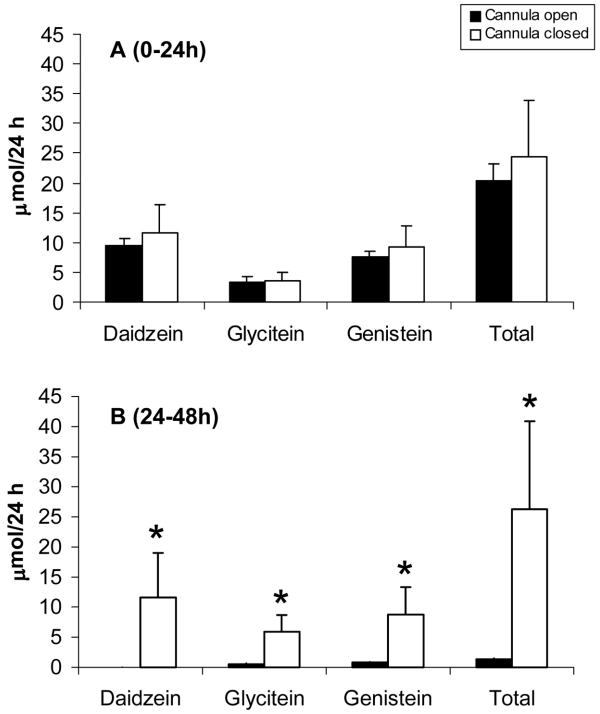

Figure 3. Urinary excretion of IFN equivalents in swine after feeding a corn–soy test diet (study number two). Swine were fed a corn–soy test diet and urine was collected for 48 h. Urinary excretion of IFN during the initial 24 h period after consuming the test diet (Panel A) was not significantly different when ileal canulae were open (0–16 h) or closed. From 24 to 48 h after feeding the test diet (Panel B), total IFN equivalents excreted in urine from swine when ileal canulae were closed after consuming the test diet were markedly greater than that when ileal canulae had been opened from 0 to 16 h. Data are expressed as means ± SEM. The presence of an asterisk above the open bars indicates that the quantity of IFN equivalents in urine when canulae were closed was significantly ($p < 0.05$) greater than when canulae were open.

When canulae remained closed throughout the 48 h collection period in the second study, urinary excretion of total and individual IFN were not significantly different between day 1 (0–24 h postingestion) and day 2 (24–48 h postingestion) (Figs. 3a and b). In contrast, when ileal canulae were opened from 0 to 16 h after ingesting the test diet, urinary excretion of total IFN (1.3 ± 0.1 μmol), and individual IFN aglycone equivalents on day 2 were significantly ($p < 0.01$) lower than that excreted in urine on day 1.

Table 5. IFN metabolites in urine of swine after ingesting the corn-soy test diet (study number two)

Metabolites[a)]	μmol ± SEM	Range, μmol	Producers[b)]
DHD	13.7 ± 10.1[AB]	0.03–31.9	4/4
DHG	28.9 ± 21.7[A]	0.03–65.9	4/4
O-Dma	0.45 ± 0.2[B]	nd[c)]–0.7	3/4
Equol	29.6 ± 16.3[A]	0.05–48.1	4/4

a) Data for urine ($N = 4$) are means ± SEM during 48 h collection with ileal canulae closed (fully functional GI tract). Means that do not share a common uppercase letter as superscript differ significantly ($p<0.05$, Fisher *F*-test). While the differences between the individual IFN metabolites failed to achieve statistical significance under the Bonferroni posthoc test, the Fisher-protected LSD test indicated significantly higher amounts of urinary DHG ($p = 0.016$) and equol ($p = 0.015$) than *O*-Dma.
b) Number of animals producing detectable amounts of the indicated metabolites.
c) nd: Not detected.

DHD, DHG, and equol were present in urine from all animals, although there was relatively large variation in production of these metabolites among animals (Table 5). Mean amounts of urinary DHD, DHG, and equol were similar. *O*-Dma was detected in urine from three of the four animals in markedly lower quantities than equol and DHG ($p = 0.015$ and 0.016, respectively, Fisher-protected LSD test). Metabolites from the daidzein and genistein families accounted for approximately 12 and 9%, respectively, of their respective aglycone equivalents in the test soy diet. 6-Hydroxy-*O*-Dma was not detected in any of the swine urine samples. The amounts of daidzein and genistein in ileal effluent were not significantly correlated ($r^2 \leq 0.18$) with their respective metabolites in urine. The metabolite profile in urine from animals with canulae open after feeding was not determined due to failure to save aliquots after assaying IFN aglycone equivalents.

4 Discussion

Although it is recognized that dietary IFN glucosides must be converted to aglycones prior to absorption, the relative contributions of microbial and brush border enzymes for this conversion remain controversial. The present study offers insight into the relative participation of the small and large intestines in the absorption and metabolism of soy IFN in swine. To our knowledge, this is the first study to use ileal-canulated swine to address intestinal metabolism and absorption of soy IFN. One advantage of this model is that feeding studies were conducted using a crossover design. Additionally, the animals demonstrated no signs of aversion to the corn–casein and corn–soy diets used in this study.

The marked deglycosylation of IFN glucosides before reaching the ileal canula suggests that the small intestine is the predominant site for hydrolysis of IFN glucosides in swine. We also have observed extensive small intestinal hydrolysis of IFN glucosides in human subjects with ileostomies [30]. Similarly, Walle *et al*. [32] found that ingested quercetin glucosides were completely hydrolyzed to aglycones prior to absorption in human subjects with ileostomies. Conversion of IFN glucosides to aglycones may be mediated by the action of the brush-border enzyme lactase-phlorizin hydrolase or microbial β-glucosidases [16–18]. Bacterial species with significant β-glucosidase activity include *Enterococci* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacteria* spp. [6]. Significant numbers of *Enterococci*, *Lactobacilli*, and *Bifidobacteriae* reside in the small intestine of swine [33–36].

The presence of small quantities of the IFN microbial metabolites DHD, DHG, 6-OH-*O*-Dma, and equol in ileal effluent further supports the likelihood of microbial metabolism of dietary IFN within the small intestine of swine. In contrast, in a previous study we did not detect IFN microbial metabolites in ileal effluent from human subjects with ileostomies following consumption of a soy-containing meal [30]. The presence of IFN microbial metabolites in the urine of human subjects with fully functioning gastrointestinal (GI) tracts, and the absence of these compounds in the urine from the majority of human subjects with ileostomies, supports the central role of the large intestine in generating IFN microbial metabolites in humans [30]. The differences in intestinal metabolism of IFN between human and swine may be due to a greater microbial density and/or diversity of species in the small intestine of pigs compared to humans.

Another interesting observation in the present study was the lack of apparent impact of ileal canula position (open *vs*. closed) on the IFN content in enzyme-treated urine collected during the initial 24 h period after ingesting the soy test diet. This suggests absorption of a portion of the ingested IFN in the small intestine. In contrast, IFN equivalents in enzyme-treated urine collected 24–48 h after test diet were significantly lower when ileal canulae were open for 16 h compared to closed after ingesting the test diet. This suggests that a portion of the dietary IFN entering the large intestine were absorbed. It is possible that total IFN in the ileal effluent were underestimated since our method of analysis would not detect phase II metabolites of the absorbed aglycones present in bile and effluxed from the intestinal epithelium into the lumen [9–11]. It should be noted that ileal T-canulation may not facilitate 100% collection of chyme. A small fraction of chyme may have been transferred to the large intestine when the ileal canulae were open as only 85% of the chromium oxide marker was present in collected chyme. This recovery is similar to that previously reported (72–106%) for swine with ileal canulae [37].

The relative amount of ingested IFN recovered in swine urine collected for 48 h after feeding with canulae closed

(6.5 ± 3.1%) was much lower than typically reported for humans (*i.e.*, 20–50%) [38]. It is particularly noteworthy that the mean quantities of microbial metabolites (9.3 ± 6.2% of ingested IFN equivalents) in 48 h urine from swine were greater than their respective aglycones. This differs from humans for which urinary excretion of IFN aglycone equivalents is higher than their respective metabolites [5, 27, 39, 40] and also may explain the relatively low recovery of daidzein, genistein, and glycitein equivalents in swine urine. Because we did not collect urine from the pigs beyond 48 h after ingestion of the soy test diet, the observed elimination of approximately 16% of ingested IFN as aglycone equivalents and microbial metabolites in urine may underestimate urinary losses of absorbed compounds.

The typical corn–soy diet fed to swine provides high amounts of IFN with respect to the amounts typically consumed by humans [41]. It is possible that chronic intake of high amounts of IFN is associated with increased metabolism of GI IFN in swine compared to humans. However, consumption of 100 mg/day of IFN from soy foods or supplements for ≥1 month did not alter equol production or the bioavailability of IFN in humans, suggesting limited impact of chronic soy and/or IFN consumption on intestinal microbial populations that metabolize IFN [42, 43].

We are aware of three previous reports regarding IFN bioavailability in swine. Kuhn *et al*. [44] measured plasma daidzein, genistein, and equol in swine (at 163 days of age) fed diets containing either soybean meal or alcohol-extracted soy protein concentrate. Direct comparison with our study is not possible since the investigators did not report urinary IFN. However, it is interesting to note that genistein was the predominant plasma IFN regardless of the diet. Furthermore, plasma concentrations of equol in pigs fed either diet were similar despite the markedly different quantities of daidzein in the two diets. Lundh [45] reported that the amount of equol in swine urine was almost threefold that of daidzein (22 *vs*. 6%, respectively) 8 h after feeding a red clover meal containing 88 mg daidzein equivalents. The amounts of daidzein and equol in 48 h urine samples for swine in our study represented 6.2 ± 3.2 and 7.9 ± 4.3%, respectively, of the ingested daidzein equivalents. The difference may be associated with feeding red clover *versus* roasted soy as the source of IFN in the two studies. In contrast with our data and that of Lundh [45], it was recently reported that daidzein concentrations were 15 times greater than equol in urine from female piglets fed human infant formula containing soy protein isolate [46]. The newborn pigs were weaned to the soy protein isolate formula at 2 days of age, whereas the swine in our study were 3 months of age and maintained on the standard corn–soy diet from weaning to initiation of the experimental study. It also is interesting that Gu *et al*. [46] found that urinary *O*-Dma was five times greater than equol in the infant pigs, whereas urinary equol was much greater than *O*-Dma in our study. This supports the likely influence of the markedly different GI microflora in newborn and weaned swine on production of IFN metabolites. Age, gender, and food matrix also have been reported to moderately affect urinary excretion of IFN in humans [47]. Other factors such as fermentable carbohydrate in the diet also may influence the production of IFN metabolites [48].

In summary, the results from this study demonstrate that the small intestine is the predominant site for deglycosylation of dietary IFN glucosides in weaned swine. Although both the small and large intestine participate in the absorption of dietary IFN, the small intestine appears to have a key role in generating the bioavailable aglycones. Because the profile of urinary IFN and their metabolites observed in our study differs from that reported previously in humans, swine may not represent an ideal model for investigating the bioavailability of dietary IFN. However, the extensive microbial metabolism of IFN observed in the present study suggests that swine may be particularly useful for studies examining microbial metabolism of dietary IFN and post-absorptive metabolism of these metabolites. Further studies with swine that address the pharmacokinetics of IFN transport and metabolism, including enterohepatic circulation, are warranted.

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