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Intestinal Microbiota Contribute to the Endogenous Formation of Thiouracil in Livestock

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ABSTRACT: In recent years, the frequent detection of the banned substance thiouracil (TU) in livestock urine has been related to its endogenous formation following consumption of glucosinolate-rich Brassicaceae crops. Besides, TU residues have been recovered in these crops upon plant-derived myrosinase hydrolysis. Through in vitro bovine and porcine static digestive simulations, the influence of gastrointestinal digestion of Brassicaceae-derived matrixes on TU formation was assessed. Following derivatization and LC-MS^2 analysis, TU was detected in colonic suspensions with traditional rapeseed, coarse colza "00" meal, cauliflower, and broccoli ranging from 3.47 to 30.96 μg kg^{-1} (bovine) and from 3.55 to 26.34 μg kg^{-1} (porcine). In stomach and small intestinal fluids, TU remained unfound, whereas upon rumen simulation TU was detected for coarse colza "00" meal (4.43 μg kg^{-1}) and grounded traditional rapeseed (0.35 μg kg^{-1}). The origin of this detection was investigated through filter-sterilizing and autoclaving the fecal inoculum causing a significant decrease in TU concentration, thereby reinforcing the possibility of an active bacterial involvement, which however was characterized with a high interanimal variation. In conclusion, these results support the previously proven endogenous origin of TU and acknowledge the active role of the gastrointestinal bacteria in TU formation, through production of an extracellular component.

KEYWORDS: *endogenous thiouracil, microbiota, Brassicaceae, in vitro static digestion simulations, LC−MS^2, livestock*

INTRODUCTION

In recent years, endogenous prevalence of thiouracil (TU) has been reported in urine of livestock upon ingestion of glucosinolate-rich crops belonging to the Brassicaceae family.[1] This has been a staggering discovery, since the European Union banned the use of thyreostatic drugs in livestock for fattening purposes in 1981. Thiouracil was until recent always considered as a synthetic, orally active thyreostatic drug, which upon administration inhibits the thyroid gland by decreasing the production of thyroid hormones: thyroxin and triiodothyronine.[2]

In livestock, illegal TU administration pairs with a desired weight gain resulting from water retention in the digestive tract and edible tissues, which obviously degrades meat quality.[3,4] Because of the European Union banishment on thyreostatic drugs, the rightful detection of their abuse is crucial.[5] This would be the case not only for residue analysis, but also for public health concerns, because thyreostats have been shown to exert teratogenic and carcinogenic effects, given that thiouracil is a 2b-categorized substance.[6]

To allow discrimination between the illicit use of TU and possible feed related TU-levels, a need for mechanistic knowledge arose. Not surprisingly, the family of the Brassicaceae was singled out as one of the most probable contributors to endogenous TU formation, as they are not only known for their high glucosinolate content, but also comprise a precursor for naturally goitrogenic substances, such as oxazolidine-thiones and thiocyanates. These breakdown products can be naturally formed upon plant cell disruption (e.g., grounding, chewing, freezing) when the endogenous β-thioglucosidase, myrosinase or thioglucoside glucohydrolase (E.C 3.2.1.147), is freed from the plant vacuoles, causing hydrolysis of the available glucosinolates.[7−9] As a result, several bioactive breakdown products (isothiocyanates, nitriles, thiocyanates, sulphates, epithionitriles, oxazolidine-2-thiones) are formed depending on the environmental conditions,[10,11] resulting in derivates with natural thyreostatic properties.[11,12]

Nowadays, growing evidence points toward the likelihood of low-level TU being endogenously formed upon digestion of the secondary plant metabolites, glucosinolates, and their degradation products.[1,13] In an attempt to confirm the possible link between glucosinolates and TU, the research by Vanden Bussche et al.[13] demonstrated that plant-derived myrosinase hydrolysis of glucosinolate-rich Brassicaceae feed and food matrixes resulted in TU detection. Thereupon the natural status of TU in the urine of various mammalians (livestock (bovine, porcine, ovine, etc.), domesticated animals (canine), and humans) was investigated. The animals displayed traces of TU, below 10 μg L^{-1} without any diet control,[14] as for humans, for whom the influence of a controlled Brassicaceae diet was investigated; TU was retrieved in 66.7% of the samples.

Besides plant-derived myrosinase hydrolysis, bacterial hydrolysis resulting from intestinal microbiota during digestion has

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also been linked to cause glucosinolate degradation.[9,15,16] For example, the hydrolysis of sinigrin, an aliphatic glucosinolate, was shown to be mediated by the human intestinal bacterium *Bacteroides thetaiotamicrometer* in gnotobiotic mice.[17] Several bacterial strains in animals have also been investigated for their glucosinolate degrading activity. For example, a *Lactobacillus* strain was identified capable of inducing a myrosinase-like enzymatic activity in vivo.[18] However, to the best of our knowledge, no research concerning the probability of TU being formed from glucosinolates-rich Brassicaceae crops upon bacterial digestion has been performed.

Consequently, the focus of the present study was to establish the potential role of the intestinal microbiota in TU formation upon Brassicaceae digestion in animals. This was performed by conducting in vitro bovine and porcine gastrointestinal static digestive simulations (stomach, small intestine, large intestine). Additionally, the difference between mono- and polygastric animals was evaluated by incorporating digestion at the ruminal level. Various Brassicaceae feed and food matrixes were evaluated by this approach.

MATERIALS AND METHODS

LC–MS^2 Reagents and Chemicals. The chemical standard 2-thiouracil (TU) was obtained from Sigma-Aldrich (St. Louis, MO). The deuterated internal standard for TU, 6-propyl-d_5-2-thiouracil (PTU-d_5),[19] was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Stock solutions of the chemical standards were prepared in methanol at a concentration of 200 ng μL^{-1}. Working solutions were diluted in methanol to 1 ng μL^{-1} for PTU-d_5 and 1, 0.1, and 0.01 ng μL^{-1} for TU. Solutions were stored in dark glass recipients at 4 °C. Reagents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification steps, and of Optima LC/MS grade for LC–MS application (Fisher Scientific UK, Loughborough, UK), respectively. The derivatization reagent, 3-iodobenzyl bromide (3-IBBr, Sigma-Aldrich, St-Louis, MO), was prepared extemporaneously (2 mg mL^{-1} methanol). The phosphate buffer, made up of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 (Merck, Darmstadt, Germany) in deionized water, was prepared and adjusted to pH 8.

Feed and Food Samples. Feeds and foods affiliated to the Brassicaceae family were selected, as they are known for their high glucosinolate content. The selected representatives were traditional rapeseed (*Brassica napus* L. partim Napoleon, Institute for Agriculture and Fisheries Research (ILVO), Melle, Belgium), coarse colza "00" meal, broccoli (*Brassica oleracea* L. convar. botrytis L. var. cymosa), and cauliflower (*Brassica oleracea* L. var. botrytis L. subvar. cauliflora) (local Belgian produce). Additional pretreatments (Table 1) were applied to the feed and food matrixes, consisting of various grounding and inactivation steps, resulting in nine treatment groups in total. Grounding required the use of a mortar and for homogenization the use of a kitchen blender. To maintain glucosinolate concentrations, scientific reports mention limiting the water content as crucial to avoid microbial growth and prevent endogenous myrosinase activity. Storage temperatures of −20 or 25 °C are advised at which Brassicacea seed meals can be preserved for 30 months and presumably much longer providing they are protected from exposure to moisture conditions that promote microbial growth.[20] Therefore, all samples were ultimately freeze-dried and stored at −20 °C in plastic containers. In this way, obtained TU concentrations for different feeds could be bilaterally compared based upon their dried weight concentrations.

Table 1. Different Pretreatments Applied to the Brassicaceae Representatives: Traditional Rapeseed, Coarse Colza "00" Meal, Broccoli, and Cauliflower

	Brassicaceae foods and feeds			
treatments	traditional rapeseed	coarse colza "00" meal	broccoli	cauliflower
2 h at 100 °C	+			
5 min at 100 °C			+	+
grounding	+			
homogenizing		+	+	+
freeze-drying	+	+	+	+

Preparation of Buffers and Broths. Digestion (stomach, small, and large intestine) and fecal inoculum buffers were prepared in ultrapure water and autoclaved for 15 min, at 121 °C and 1 atm to ensure the absence of bacterial growth. Procedures following autoclavation were all performed in a laminar flow cabinet to exclude bacterial contamination.

Fecal inoculum buffer contained K_2HPO_4 (8.8 g L^{-1}), KH_2PO_4 (6.8 g L^{-1}) (Merck, Darmstadt, Germany), and sodium thioglycolate (1.0 g L^{-1}) (Sigma-Aldrich, Steinheim, Germany).

The stomach buffer consisted of $KHCO_3$ (10.0 g L^{-1}) (Sigma-Aldrich, Steinheim, Germany) and NaCl (5.8 g L^{-1}) (Merck, Darmstadt, Germany), which once dissolved was brought to a pH of 1.5 ± 0.1 by adding 6 M HCl (37%) (Merck, Darmstadt, Germany). A universal pH-indicator (Merck, Darmstadt, Germany) was used as an additional control. The enzyme, pepsin (0.32 g L^{-1}) (E.C. 3.4.23.2) (Sigma-Aldrich, St-Louis, MO), was filter-sterilized (Filter Millex-GV, 0.22 μm, Millex, Cork, Ireland) and added to the stomach buffer after autoclavation of the latter, to prevent denaturation of the enzyme.

Small intestinal buffer was made up of $NaHCO_3$ (12.6 g L^{-1}) (Merck, Darmstadt, Germany), Oxgall (6.0 g L^{-1}) (Becton, Dickson and Co., NJ), and pancreatin (0.9 g L^{-1}) (Sigma-Aldrich, St. Louis, MO).

The ruminal phosphate buffer comprised NH_4Cl (1.4 g), $Na_2HPO_4{\cdot}12H_2O$ (28.8 g), and NaH_2PO_4 (6.2 g), which were dissolved in 1 L of distilled water with a consequent pH of 7 (Merck, Darmstadt, Germany).

The BHI (Brain Heart Infusion) broth (Oxoïd, Hampshire, England) constituted out of a ready to use powder, which had to be dissolved in ultrapure water (37 g L^{-1}). L-Cysteine (0.5 g L^{-1}) (SAFC Supply Solutions, St. Louis, MO) was added for improvement of the anaerobiosis.[21] BHI broth was used to grow the fecal bacteria for 24 h, which provided the needed macrobiotic culture for the large intestinal digestion. Simulator of the Human Intestinal Microbial Ecosystem broth (SHIME) was used as a medium for the actual large intestinal digestion. This medium consisted of (g L^{-1}): arabinogalactan, 1; pectin, 2; xylan, 1; mucin, 4 (all from Sigma-Aldrich, St. Louis, MO); potato starch, 3 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); glucose, 0.4 (Merck, Darmstadt, Germany); yeastcell extract, 3 (AppliChem GmbH, Darmstadt, Germany); peptone, 1 (Oxoïd, Hampshire, England); L-cystein, 0.5 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).[22] Both SHIME and BHI broths were reboiled before use, regaining anaerobicity.

Digestion Protocol. The various in vitro digestive simulation protocols applied during this study were adapted from scientific literature on human and animal in vitro models, reporting simulation of the upper and lower gastrointestinal tract of bovines and porcines.[23,24]

Fecal Inoculum. To set up a large intestinal digestion simulation, a fecal inoculum is required. For this purpose, fresh fecal matter was collected from adult female nonpregnant cows and sows from Ghent University, Faculty of Veterinary Medicine and from the Institute of Agricultural and Fisheries Research (ILVO, Melle, Belgium). The animals were housed according to animal welfare requirements, keeping them on a maintenance diet and antibiotic-free. The feces were kept at room temperature during transport before further processing was applied.

Fecal slurry was obtained by addition of 1/5 (S/L) fecal phosphate buffered saline, which subsequently was homogenized in a stomacher for 10 min. The suspension was transferred into 50 mL falcon tubes and centrifuged at 500g for 2 min. To the supernatant glycerol (99.5%) (Analar Normapur, Fontenay-sous-Bois, France) was added a

cryoprotectant at a 20% (v/v) ratio, which was gently mixed under atmospheric conditions, before storage at −80 °C.[25]

Ruminal Inoculum. The ruminal fluid for the rumen simulation was collected before the morning feeding from three permanently cannulated cows (ILVO, Melle, Belgium) fed a hay-based diet ad libitum. Immediately after collection, samples were filtered through a metallic sieve (mesh width 1 mm) and, under CO_2 flushing, diluted 5-fold with a phosphate buffer at 39 ± 1 °C. The rumen fluid and buffer mixture was kept saturated with CO_2, at 39 ± 1 °C at all times.

Stomach, Small Intestinal, and Large Intestinal Simulations. Autoclaved penicillin flasks were supplemented with feed (3 g) or food (4 g) samples following a 1/25 (S/L) ratio. The stomach digestion was simulated through addition of acidified stomach buffer (pH 1.5 ± 0.1) in a 1/10 (S/L) ratio to the samples. The flasks were capped and incubated at 37 ± 1 °C, 150 rpm for 2 h. Next, 5 mL g^{-1} of pancreatic fluid was added and incubated for 4 h to simulate the small intestine. Next, the fecal inoculum was thawed and diluted with reboiled BHI in a 1/9 (S/L) ratio in an autoclaved dark penicillin flask. Before the flasks were incubated, anaerobic conditions were established using a N_2 flush system for 1 h, at 1 bar alternating every 2 min with vacuum suction. The flasks were then incubated for 24 h. This incubated bacterial inoculum was used for the large intestine digestion simulation, using 5 mL g^{-1} in combination with 5 mL g^{-1} boiled-up SHIME or BHI medium. Both were added to the small intestinal fluid flasks and flushed for 1 h prior to incubation (72 h) to ensure proper growing conditions for the anaerobic bacteria.

Sampling of the different digestion phases was done as secure and standardized as possible using syringes, causing as little disturbance as possible to the bacterial environment. Stomach, small intestinal, and large intestinal digestion fluids (at 0, 24, 48, 72 h of incubation) were gently stirred to homogenize before sampling.

Ruminal Digestion Simulation. The rumen digestion protocol consisted of 0.25 or 1.0 g of freeze-dried feed, which was contained in a transparent 125 mL penicillin flask. Next, the flasks were flushed under alternating CO_2-flow and vacuum suction for anaerobiosis.[26,27] Into every flask was added 25 mL of a buffer−rumen fluid mixture was added. The internal standard ethane for GC analyses was also added (1 mL) to the flasks, which were then incubated at 39 ± 1 °C for 24 h with an adapted RPM-program simulating ruminal contractions.

Subsequently, samples were put in an ice bath causing a thermal shock to the bacteria inhibiting further growth. Gases (ethane, methane, hydrogen), pH, short chain fatty acids (SCFAs), and TU were then measured. A duplo blank sample (without feed) at the beginning of the digestion and another duplo blank sample, which endured the 24 h incubation, were required to assess SCFA and TU production. Combining both values allowed quantification as blank levels could be subtracted from the measured values.

Sample Extraction and Purification. Prior to the analysis of the digestion samples, sample preparation and cleanup was needed. This procedure has been described elsewhere.[28]

Briefly, 5 mL of phosphate buffer (pH 8) was added to 1 mL of digest. The internal standard (PTU-d_5) was added at 50 μg kg^{-1} followed by 100 μL of a methanolic derivative solution containing 3-iodobenzyl bromide (2 mg mL^{-1}). Upon 10 min sonication, the derivatization was allowed to proceed in the dark at 40 ± 1 °C for 1 h. Afterward, the pH of the reaction mixture was adjusted to 3.6 ± 0.1, and different liquid liquid extraction steps with 3, 2, and 2 mL of diethyl ether were applied. Once evaporated to dryness under a gentle N_2 stream (2 bar, 50 ± 1 °C), the samples were redissolved in 100 μL of CH_2Cl_2 and 300 μL of cyclohexane. Further sample cleanup consisted of solid-phase extraction with silica cartridges conditioned with cyclohexane and eluted with a mixture of hexane/ethyl acetate 40:60 (v/v). Again, N_2 evaporation was applied. Samples were redissolved in 160 μL of 50:50 (v/v) 0.5% acetic acid and methanol.

Instrumentation. *Control Parameters: Short Chain Fatty Acids and pH.* Colonic samples of the full digestion were assessed for their short chain fatty acid (SCFAs) content. Fatty acid extraction consisted of a 2 mL sample to which 500 μL of aqueous H_2SO_4 1:1 solution (Sigma-Aldrich, St. Louis, MO), 0.4 g of NaCL (Merck, Darmstadt, Germany), 400 μL of internal standard 2-methylhexane (Sigma-Aldrich, St. Louis, MO), and 2 mL of diethyl ether (Merck, Darmstadt, Germany) were added. Samples were shaken for 2 min and centrifuged for 3 min at 341*g*. Next, the supernatants were collected for GC analyses on a Di200 (Shimadzu's Hertogenbosch, Netherlands) with an EC-1000 Econo-Cap capillary column (1.2 μm × 25 m × 0.53 mm) by Alltech (Laarne, Belgium). A flame ionization detector (FID) and a Delsi Nermag 31 Integrator were used. N_2 was used as a carrier gas with a flow of 20 mL min^{-1}. Column temperature was set at 130 °C, and the injector temperature was at 190 °C.

The rumen samples were analyzed in the Laboratory for Animal Nutrition and Animal Product Quality of the Faculty of Bioscience Engineering (Melle, Belgium) according to the specifications mentioned in Van Ranst et al.[29]

The pH was measured in both colonic and ruminal samples with an electrode pH-meter (SevenEasy pH, Mettler Toledo AG, Scherzenback, Switzerland), which was calibrated before use.

Liquid Chromatography Multiple Mass Spectrometry (LC−MS^2). Detection of thiouracil was achieved with a liquid chromatograph coupled to a linear ion trap mass spectrometer.[13] A Finnigan Surveyor LC-system (Thermo Electron, San Jose, CA) was combined with a Symmetry C18 column at 30 °C (5 μm × 150 mm × 2.1 mm, Waters, Milford, MA) running on a 0.5% acetic acid (A) and methanol (B) 50:50 solvent combination at 0.3 mL min^{-1}. The linear gradient passed off as follows for 35 min: A/B 50:50 for 3 min, increasing to 0:100 in 17 min, and finally re-equilibrating for 10 min at 50:50. The linear ion trap mass spectrometer LTQ (Thermo Electron, San Jose, CA) was fitted with a heated electrospray ionization probe (HESI) operating in the negative ion mode and positioned on 0.5; C; 0 (front-to-back position (micrometer lines); probe depth (probe depth line); side-to-side position (+1 to −1 marks)). Applied working conditions were as follows: source voltage at 5 kV; capillary voltage at −50 V; tube lens voltage at −128.04 V; vaporizer and capillary temperature at 250 and 275 °C; sheath and auxiliary gas at 30 and 5 arbitrary units (au), respectively. Measured transitions are reported in Table 2.

Table 2. Monitored Transitions by the LC−MS^2 for 2-Thiouracil and Its Internal Standard, 6-Propyl-d_5-2-thiouracil

analyte	$[M-H]^-$	product ions	collision energy (eV)
2-thiouracil	343	182; 215; 309	44
6-propyl-d_5-2-thiouracil	390	127; 262; 356	30

Quality Assurance. Preceding the LC−MS^2 analysis, a standard mixture of the target compound and the internal standard were injected to check the operational conditions of the LC−MS^2 device. Identification of TU was based on retention time relative to the internal standard, and the ion ratios of the product ions according to the Commission Decision 2002/657/EC.[30] Every feed or food digestion simulation was conducted in 3-fold, and the internal standard was added prior to cleanup.

Standard Addition. Quantification of TU in digestion samples was performed using the standard addition approach as described in Commission Decision 2002/657/EC.[30] Each sample was divided over two vials with analogous mass and volume. One aliquot, the unknown, was added with a mixture of 0.5% acetic acid and methanol (v/v) 50:50, and the other, the known, was enriched with an equal amount of analyte (thiouracil). The concentration of the addition solution was previously determined by analyzing thiouracil in digest samples and fitting these in a calibration curve in digestion buffer. Finally, quantification was established through evaluation of the area ratios of the known and unknown samples using the standard addition approach.[13]

Data Handling. All data processing was performed with XCalibur 2.0.7 (Thermo Fisher Scientific, San Jose, CA). Statistical testing (Student's-*t* test, ANOVA) was carried out with Sigmaplot 12 (Systat Software GmbH, Erkrath, Germany) to assess significance (p-value < 0.05) of the type of matrix (feed and food) and the applied treatments

on the recovered TU yields. Normality and equal variance were prerequisited.

RESULTS AND DISCUSSION

Porcine Digestion. In the stomach and small intestinal fluids of the porcine simulation, no thiouracil could be recovered within the methods detection limits. The probable absence or the negligible yield of TU in the stomach and the specific acidic conditions at those stages might explain these findings. Reportedly, an in vitro digestion study in pigs also claimed natural goitrogens, including oxazolidine-thiones (OZTs), to fail to be detected under pepsin and small intestinal conditions.[31] The glucosinolate loss under in vitro peptic and small intestinal conditions in pigs ranged between 3−23% and 7−28%, respectively. Besides, the maximum activity of myrosinase occurred at moisture levels between 15% and 40% and a pH range of 3.5−8.0,[32] implying that the effect of plant enzyme myrosinase in the stomach is most probably negligible.

Analysis of colonic digestion samples however did allow TU detection (Figure 1). From these data, it may be concluded

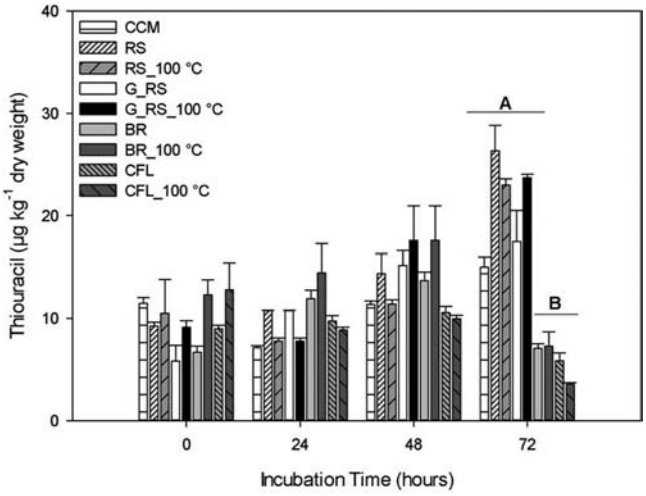

Figure 1. Thiouracil yield (μg kg^{-1} dry matter ± standard error) of various foods and feeds in the large intestinal fluid of an in vitro porcine model (CCM, coarse colza "00" meal; RS, rapeseed; RS_100, rapeseed, 2 h at 100 °C; G_RS, grounded rapeseed; G_RS_100, grounded rapeseed, 2 h at 100 °C; BR, broccoli; BR_100, broccoli, 5 min at 100 °C; CFL, cauliflower; CFL_100, cauliflower, 5 min at 100 °C). The *Napus* group (A) has a significantly (p-value < 0.05) higher TU yield than the *Oleracea* group (B) after 72 h.

that, irrespective of the pretreatment, representatives of the *Napus* group (rapeseed variants) resulted in a significantly (p-value < 0.05) higher TU formation as compared to representatives of the *Oleracea* group (cauliflower and broccoli). This difference in TU recovery between the *Oleracea* and *Napus* matrixes is with a high likelihood attributed to their difference in glucosinolate content. In parallel, evaluation of enzyme inactivation through cooking and/or grounding was performed to gain more insight in the availability and activity of the plant myrosinase. Heating at 100 °C for 2 h (*Napus* group) or for 5 min (*Oleracea* group) could lead to denaturation of the plant myrosinase and the possible degradation of glucosinolates into certain byproduct. It has been reported that unpressured heat treatments destroy myrosinase in, for example, *Brassica napus* seeds from 60 °C on and can cause glucosinolates to deteriorate and/or byproducts to evaporate.[32,33] Furthermore, grounding enables the release of glucosinolates and plant myrosinase from the feed, causing a hydrolysis reaction. This plant myrosinase has been shown to lead to TU formation in Brassicaceae feed.[13] However, grounded rapeseed showed a significant TU decrease as compared to the grounded previously cooked variant. No other significant differences were found between treated and untreated Brassicaceae matrixes, implying that the potential effect of these pretreatments did not significantly contribute to the whole of the TU formation. This coincides with the previous stomach fluid results, as in analogy the applied pretreatments also left the TU formation unaffected. Coarse colza "00" meal showed a lower TU recovery as compared to rapeseed, as it consists of "00" rapeseed, which is a rapeseed variant with already a low glucosinolate content (<25 μmol g^{-1}) to start with.[34] Besides, coarse colza "00" also undergoes grounding, hot pressing (80−140 °C), hexane extraction, and toasting during its production process, which may negatively influence further TU formation upon digestion.

During digestion, parameters including pH and volatile fatty acids were used to evaluate the proper digestion conditions. Short chain fatty acid recoveries in the in vitro large intestinal fluids approximated the known in vitro acetate > propionate > butyrate ratio, which in vivo approaches a 70:20:10 ratio in livestock.[35,36] Both volatile fatty acids and pH values were found within the physiological values for bacterial growth, which are substrate, species, and animal dependent, as described elsewhere.[35,37]

In general, for the porcine model, it may be concluded that the large intestine is the main site of TU formation. In an ulterior research,[13] identical Brassicaceae feeds and foods upon plant-myrosinase hydrolysis were analyzed for their TU content. In summary, TU was retrieved in the highest concentrations for broccoli, traditional rapeseed, and coarse colza "00" meal displayed (5.98, 1.45, and 1.59 μg kg^{-1}, based on dry weight). Other samples yielded very low TU concentrations (<1.0 μg kg^{-1}) (cauliflower, rapeseed cake) or undetectable signals (feeding cabbage, feed 30% rapeseed "00") due to background noise. When comparing these absolute concentrations, the here obtained TU concentrations upon digestion were up to a factor 10 higher depending on the matrix. This confirms the involvement of colonic digestion and, most likely, microbial fermentation in the formation of TU.

Bovine Digestion. *Rumen Digestion.* Reportedly ruminants are more tolerant to glucosinolate fermentation breakdown products at the ruminal level as compared to other animals consuming forage brassica crops.[38] On that account, the capacity of ruminal microbiota to form TU at the ruminal level upon brassicaceae digestion was evaluated. Glucosinolate fermentation was assessed through a static 24 h in vitro rumen digestion with coarse colza "00" meal, whole rapeseed, and grounded rapeseed fermentation in triplicate. Yet only when feed amounts were increased from 0.25 g dry weight (S/L = 1/100) to 1 g dry weight (S/L = 1/25), thereby matching the S/L ratio of the intestinal digestion simulations, TU was detected in the rumen. Coarse colza "00" meal showed a TU yield of 4.43 ± 0.35 μg kg^{-1} dry matter and grounded traditional rapeseed of 0.35 ± 0.01 μg kg^{-1} dry matter. For whole rapeseed, TU remained unfound in the rumen, most probably due to the low availability of this substrate to the ruminal bacteria.[39,40]

Because increasing the feed amount might saturate the ruminal digestion system by increasing the organic matter

despite the ruminal buffer mixture, the pH, produced gases, and short chain fatty acids (SCFAs) were carefully analyzed for abnormalities.[41,42] All measured values approximated the normal physiological conditions required for ruminal fermentation, that is, pH ranging from 6.0 to 6.8 and SCFA proportions within the expected ratios (i.e., acetate:propionate:butyrate between 70:20:10 and 60:30:10).[42]

Therefore, these results demonstrate the TU forming potential of the rumen microbiota under specific in vitro conditions, at relatively low concentrations (0.35 and 4.42 μg kg^{-1} dry matter) as compared to the TU yields observed in the bovine colonic simulation for coarse colza "00" meal and traditional rapeseed, respectively, 7.86 and 30.96 μg kg^{-1} dry matter.

Bovine Colonic Digestion. Results similar to those obtained in the porcine model appeared when evaluating the bovine colonic digestive suspension for TU (Figure 2). In summary,

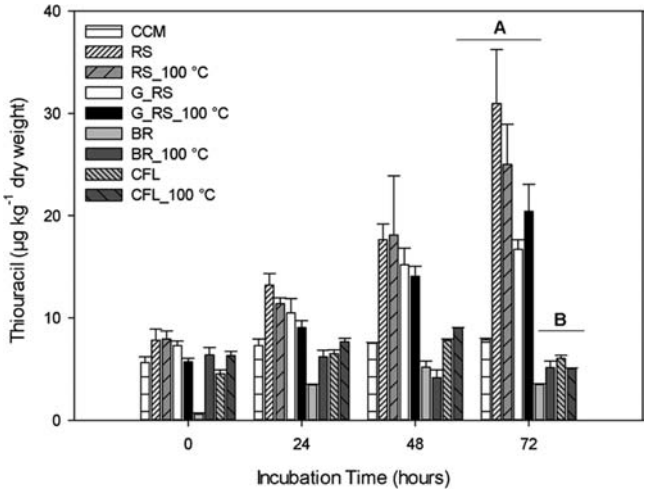

Figure 2. Thiouracil yield (μg kg^{-1} dry matter ± standard error) of various foods and feeds in the large intestinal fluid of the in vitro bovine model (CCM, coarse colza "00" meal; RS, rapeseed; RS_100, rapeseed, 2 h at 100 °C; G_RS, grounded rapeseed; G_RS_100, grounded rapeseed, 2 h at 100 °C; BR, broccoli; BR_100, broccoli, 5 min at 100 °C; CFL, cauliflower; CFL_100, cauliflower, 5 min at 100 °C). The *Napus* group (A) has a significantly (p-value < 0.05) higher TU yield than the *Oleracea* group (B) after 72 h.

the *Napus* group showed a significantly higher TU forming capacity as compared to the *Oleracea* group, with coarse colza "00" meal exerting a significantly low TU recovery as compared to the other traditional rapeseed treatment groups.

The bovine model seemed equally unaffected by the inactivation treatments as even less significance (p-value > 0.05) was found between treated and untreated matrixes than for the porcine model. Therefore, the observed TU yield variation is most probably due to variation in the microbial colonic population of these two species.

The pH, with a reported in vivo median value of 7.4,[37] and short chain fatty acids (SCFAs), with a reported 70:20:10 ratio,[36] were also observed in the bovine model. All obtained values were found within the excepted physiological ranges for digestion and bacterial growth.[35,37]

In general, for bovines the large intestine may, in line with porcines, be considered as the main site of TU formation, with a, respectively, minimal influence designated to the rumen.

Uncovering the Mechanism of TU Formation in the Colon. For both the porcine and the bovine model, TU formation was only observed in the highly microbially populated areas of the digestive tract, that is, the large intestine (for both the porcine and the bovine model) and to a lesser extent in the bovine rumen. Therefore, the assumption was made that gastric and small intestinal digestions do not significantly (p-value > 0.05) alter the ultimate large intestinal TU concentration. Additional experiments were designed to further investigate the nature of TU formation in the large intestine.

Up until now, the equilibrated SHIME medium had been used, which is specifically designed to maintain and not immediately boost the intestinal microbiota.[22] Therefore, another broth was chosen to significantly enhance bacterial growth. In this case, Brain Heart Infusion (BHI) broth, which is a liquid medium, very rich in nutrients, and suitable for the cultivation of a broad range of bacteria, was the recommended choice.[43] In the presence of BHI broth, traditional rapeseed (RS) and coarse colza "00" meal (CCM), as the highest and lowest TU producing feedstuffs from previous experiments, were incubated with the porcine and bovine inocula for a single-step large intestinal simulation. Frequent sampling (1, 3, 6, 24, 48, 72 h) allowed insight into the time scale of TU transformation throughout digestion for both species. Subsequently, a tipping point in TU formation was revealed for traditional rapeseed at 24 h for the bovine model and at 48 h for the porcine model (Figure 3). Coarse colza "00" meal, on

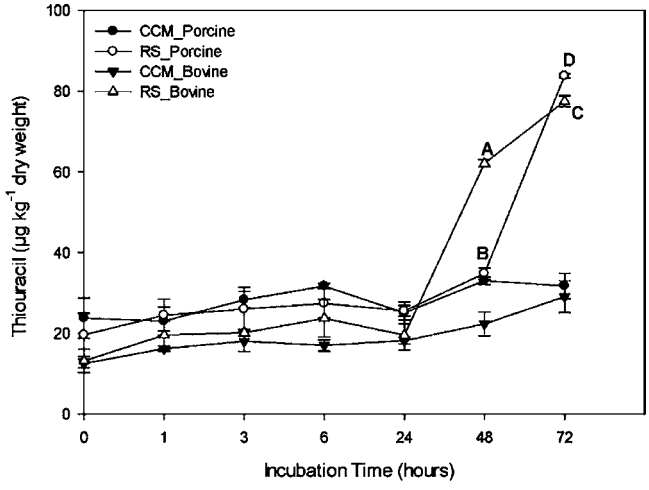

Figure 3. Representation of the TU production (μg kg^{-1} dry matter ± standard error) upon digestion of coarse colza "00" meal and traditional rapeseed in the bovine and porcine sole large intestine digestion. (A) demonstrates (B) significant (p-value < 0.05) increase of TU at 48 h of incubation as compared to (B), whereas at 72 h (C) shows a slight, but significant, TU decrease as compared to (D).

the other hand, showed a steady, but slow increase in TU concentration. The TU concentrations obtained through this experimental setup were twice as high as compared to the previous full digestion approach, confirming the positive influence of the BHI medium on bacterial growth and TU formation. The difference in tipping point between the porcine and bovine model is most likely influenced by interspecies microbial diversity and the subsequent species-specific fecal metabolite profile.[44,45] Human, primate, and nonprimate mammalian fecal microbial populations have been shown to

be more similar to each other within the same species than between different host species.[44] Besides, bacterial diversity increases based upon phylogeny from carnivory to omnivory to herbivory, which might explain the variations we see between the bovine and porcine model, as one is omnivore and the other herbivore. Furthermore, the digestive bacterial population is subject to diet and therefore forced to codiversify with their hosts eating behavior.[44]

Thereupon, interanimal variability was evaluated within one species (porcine), through comparison of the fecal inocula of 10 female porcines upon rapeseed colonic digestion. Eight animals originated (2−6; 8−10) from the Institute for Agriculture and Fisheries Research (Melle, Belgium) and two animals (1, 7) from the Faculty of Veterinary Medicine (Ghent, Belgium); both individual groups comprised animals of the same age, sex, and were held under equal housing and feeding conditions (Figure 4). TU production showed a significant (p-value < 0.05) interanimal variation between the porcines even when they were kept under similar housing conditions. The coefficient of variance (CV%) of the TU concentrations at the beginning and the end of the 72 h incubation amounted to 73% and 31%, respectively. These findings support the bacterial involvement in TU formation, because the interanimal differences in colonic bacterial composition and activities most probably are responsible for the observed differences in TU yield between various pigs. Microbial digestion of feed has previously been reported in the literature to engender a high interanimal variation in pigs.[46]

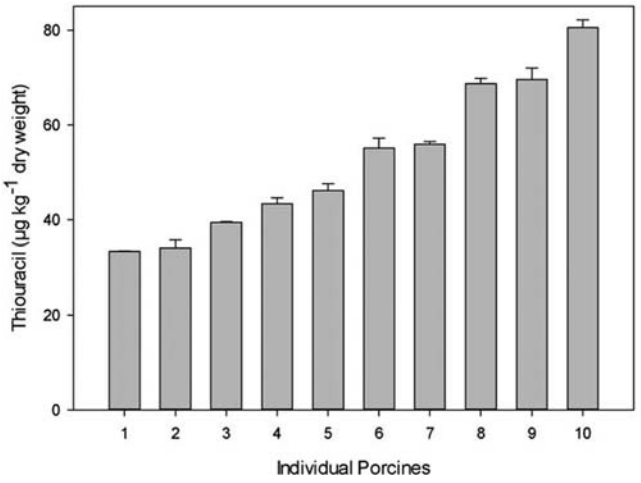

Figure 4. Interanimal variation in porcines of TU abundance (μg kg^{-1} dry matter ± standard error) upon a sole colonic simulation of the same feed (rapeseed).

Subsequently, confirmation of the mechanistic nature of the microbial formation of TU was envisaged, and, to this extent, the influence of autoclavation (121 °C, 1 atm, 15 min) and filter-sterilization of the inoculum (0.22 μm), as well as autoclavation of the rapeseed feed source (121 °C, 1 atm, 15 min) on TU detection, were evaluated (n = 3). The highest TU-producing porcine inoculum was subjected to different treatments in a colonic in vitro digestion and compared to a positive control.

Autoclavation showed no significant TU formation, whereas TU formation with the filter-sterilized inoculum and autoclaved feed were left unaffected as compared to the control (Figure 5). This indicates that bacterial contamination of feed does not play a role in the conversion of Brassicaceae feed precursors to TU. Because filter-sterilization removes all bacteria from the inoculum and still causes TU formation concurring with the positive control, the involvement of a bacterially produced extracellular secondary product, such as an enzyme involved in TU formation, may be presumed. To further justify this presumption, another setup compared a filter-sterilized inoculum to a positive control sample (n = 3). In this particular case, the inoculum was not pre-enriched for 24 h prior to the 72 h incubation, which was the case for all previous digestions. Thereby, the effect of filter-sterilization (0.22 μm) on TU formation could be evaluated, because bacteria were removed from the beginning of the incubation. After 72 h of incubation, the filter-sterilized inoculum was found to be 80% lower in TU concentration as compared to the positive control. On the basis of these results, attempts were made to evaluate whether this responsible enzyme could be the hypothesized bacterial myrosinase, alias β-thioglucosidase or thioglucoside glucohydrolase (E.C 3.2.1.147). To this end, the synthetically available glucosinolate, sinigrin, was added to colonic digestive fluids. If myrosinase were present in these suspensions, this would lead to the hydrolysis of sinigrin with loss of glucose, which then could be detected by an HPLC-ELSD system.[13] To this point, we have been unsuccessful in achieving this conversion based upon "bacterial myrosinase" in the digesta. Yet, when adding plant myrosinase extracted from *Sinapsis alba* L. seeds (white mustard) to these sinigrin enriched digesta, glucose was detected, implying that the conversion is indeed possible within this matrix. However, literature reports cell-free supernatants of *Lactobacillus agilis* cultures to be incapable of sinigrin degradation[47] and implies a cell-associated activity for "bacterial myrosinase". This cannot be correlated with our findings, as filter-sterilized precultured digesta were able to form TU in the absence of bacterial cells. Furthermore, when estimating the relative myrosinase amount, which could have been present in the digesta, based upon their TU yield, the calculated amount of bacterial myrosinase should have approximately ranged between 10 and 30 times the amount of myrosinase administered in the research by Vanden Bussche et al.[13] for recovery of the same TU amounts in identical feed

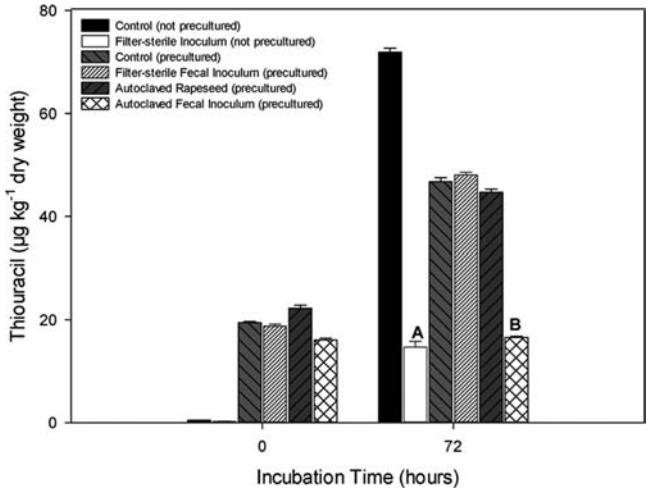

Figure 5. Representation of the effects of preculturation, autoclavation, and filter-sterilization on TU formation (μg kg^{-1} dry matter ± standard error) during a porcine colonic rapeseed digestion simulation. Treatments A and B demonstrate a significant (p-value < 0.05) TU lowering effect.

matrixes. Therefore, if bacterial myrosinase had been formed in the digestion fluids, sinigrin degradation should have taken place, subsequently causing glucose detection. These discrepancies indicate the likelihood that TU formation upon Brassicaceae digestion is independent of a myrosinase-like bacterial enzyme, but involves an unidentified bacterial enzyme or metabolite.

In conclusion, the results obtained from this study strongly imply bacteria to be involved in the gastrointestinal formation and detection of TU in livestock. Brassicaceae digestion in bovine and porcine in vitro simulations showed detectable TU levels in the large intestine for both models and in the rumen for bovines, although to a considerable lower extent. Besides, the influence of plant myrosinase was found negligible as inactivation treatments failed to significantly coherently decrease TU yields. On the other hand, the influence of the bacterial population was quite important, as different TU yields were recovered for the same Brassicaceae feed within the same species. The bovine and porcine culture also reacted differently for some feeds. The agent responsible for the TU formation upon digestion of Brassicaceae matrixes is likely to be a thermosensitive component with a cell-independent activity, which resides extracellularly after bacterial formation through a fortuitous process, which is independent of Brassicaceae presence. Further investigation regarding this mediator involved in TU formation upon Brassicaceae digestion in livestock seems desirable.

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Notes

The authors declare no competing financial interest.

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