Research Article

Investigation of Ruminant Xenobiotic Metabolism in a Modified Rumen Simulation System (RUSITEC)

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Abstract

The approval of plant protection agents requires xenobiotic metabolism and residue studies in rats, farm animals and plants (e.g., EU regulation 1107/2009) performed according to OECD guidelines. The intestinal metabolism of ruminants can produce specific residues, which must be investigated in detail. This is usually done by performing additional *in vivo* studies. The aim of the present work is to assess whether a modified *in vitro* rumen simulation system (RUSITEC) can generate this information. Rumen constituents from sheep were incubated over 8 days. The pH (6.70 ± 0.07), the redox potential (301 mV ± 30 mV), the microbial composition, and β-glucosidase activity were monitored. After an equilibration period of four or five days the fermenters were probed with ^{14}C-labelled triazole derivatives, i.e., common metabolites of azole fungicides. Only triazole-alanine was cleaved to 1,2,4-triazole, while triazole-acetic acid and triazole-lactic acid remained stable for up to 96 hours. The two glucosides octyl-β-D-glucopyranoside and polydatin, which are common residues found in plants, were both rapidly cleaved in the *in vitro* rumen system. The data shows that the modified RUSITEC system is stable, viable and maintains metabolic capacity over a longer period of time (at least 8 days). This makes many animal experiments obsolete and represents a significant contribution to the 3Rs (refine, reduce, replace). The modification of the RUSITEC system enables safe use of unlabeled but also of radiolabeled test compounds.

1 Introduction

In the development of plant protection products extensive tests are required by the approving agencies such as, e.g., the European Food and Safety Agency (EFSA) in Europe and the United States Environmental Protection Agency (US-EPA) in the US, to prove the safety of the product to the environment, animals and humans. Xenobiotic metabolism studies in rats, farm animals and plants identify and quantify the active ingredient and its related degradation products, i.e. metabolites.

The active ingredient or metabolites of the product may enter the food chain; therefore, a detailed assessment of the quality and quantity of the residues is required. The results are compared with the results of rat metabolism studies to determine the appropriate coverage of the relevant metabolites by toxicological studies in rats (EFSA, 2016). If they are not covered, additional toxicological studies are required. All xenobiotic metabolism studies are performed according to OECD TG 417 (rat; OECD, 2010), 503 and 505 (farm animals; OECD, 2007a,b), 501 and 502 (plants; OECD, 2007c,d).

Based on the outcome of these studies, specific aspects of xenobiotic metabolism in ruminants may arise: 1) Are the observed metabolites ruminant-specific and formed directly in the rumen? 2) Are ruminants able to cleave plant specific metabolites like glycosides to the respective aglycon? These questions are commonly investigated by performing additional *in vivo* studies in ruminants according to OECD 503 (OECD, 2007a), which are treated with radiolabeled test item, housed in metabolic cages for at least 5 days, and sacrificed at the end of the study.

To avoid these animal experiments in the future and contribute significantly to the 3R principle, a method to replace this type of study was developed by adapting and improving an existing method called the rumen simulation technique RUSITEC. Ruminant metabolism differs from that of other animals and man with regard to the forestomach system (anaerobic conditions) and specific contribution of microorganisms, e.g., to cellulose degradation as reported by Czerkawski and Breckenridge (1977).

A rumen simulation system (RUSITEC) is an *in vitro* system containing rumen, solid feed and buffer incubated under anaer-

obic conditions at 39°C. In the past, this method was mainly used to measure methane production (Becker, 2012) or the contribution of microbes to metabolism (Oeztuerk et al., 2005). In the present study, the original RUSITEC was further refined to make it safer and easier to use for routine analysis of xenobiotic metabolism, including handling of radiolabeled compounds. The modifications were performed with the aim to establish an *in vitro* test system to avoid extensive animal experiments to test plant protection agents (Clippinger et al., 2016).

To answer whether the observed metabolites are ruminant-specific and are formed directly in the rumen, the metabolism of triazole derivative metabolites (TDM) was investigated. Azoles are developed and produced by different companies and represent a widely used group of fungicides in agriculture. The azoles form common metabolites, i.e., triazole derivative metabolites (TDM) (Stroeher-Kolberg et al., 2014; FAO, 2007; TDMDG, 2016). The toxicological properties of TDMs and their xenobiotic metabolites are of concern to the agencies and therefore additional safety studies are required (EFSA, 2015; TDMDG, 2016).

To answer whether ruminants can cleave plant specific metabolites like glycosides to the respective aglycon, the metabolism of glucoside conjugates was investigated. Glycosides are important plant constituents (Jenner et al., 2005; Bennett and Wallsgrove, 1994) and rumen contains microorganisms with β-glucosidase activity that can potentially cleave the glycosides to the respective aglycon. Radioactively labelled octyl-β-D-glucopyranoside was used as a model compound to analyze the stability and enzymatic activity of the *in vitro* system. Polydatin, the glucoside-derivative of resveratrol, was used to study the metabolism of a non-labeled compound.

2 Animals, materials and methods

2.1 Animals

The three sheep used in this study were housed on a farm of the "*Landwirtschaftliche Untersuchungs- und Forschungsanstalt* (LUFA)" in Speyer, Germany. The animals were fed hay and concentrate, housed individually with contact to each other and bedded on straw. Animals were checked on a daily basis for any signs of discomfort by the trained staff (> 30 years experience) and inspected regularly by a veterinarian. The sheep were fitted with permanent rumen cannulas (approved by the ethics committee at *Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit* (LAVES) Reference Number. 33.4-42502-04-13A373). The animals are used to the procedure due to regular sampling for tests on animal feed, so the sampling (performed within 5 min) of the rumen fluid did not appear to stress the animals. Beside the liquid rumen, solid rumen constituents, which accumulate in the cannulas, were collected into pre-warmed isolated flasks (neoLab-Weithals-Isoliergefäß; neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany). The liquid rumen was withdrawn by aspiration and approx. 400 ml from each sheep combined into another isolated flask (neoLab-Edelstahl-Isolierflasche 1,0 l; neoLab Migge
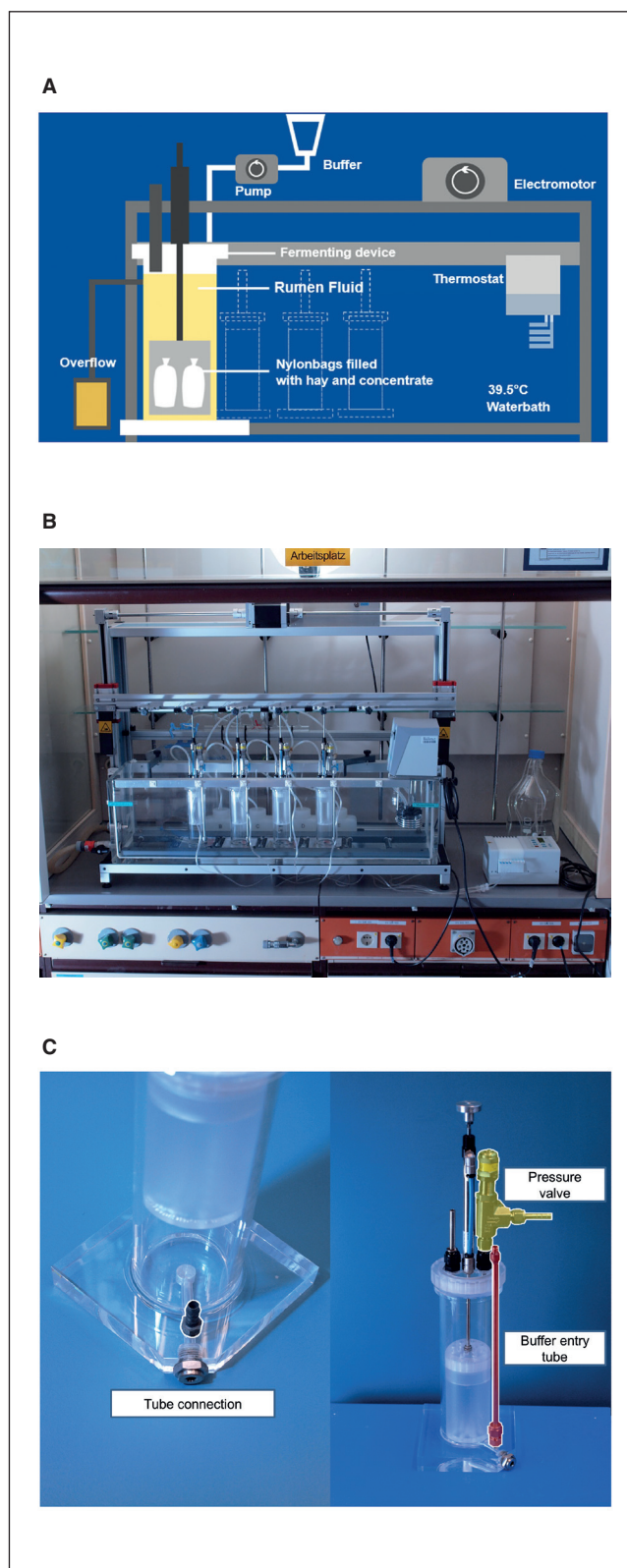

Fig. 1: The RUSITEC system

A, Schematic of the RUSITEC system; B, Photograph of the RUSITEC system; C, RUSITEC system modified by BASF.

Laborbedarf-Vertriebs GmbH, Heidelberg, Germany). Prior to being filled, all flasks were rinsed with nitrogen to enable anaerobic conditions. The transport to the laboratory generally took no more than 30 min. However, it has been observed in internal studies (data not shown) that the rumen fluid is stable also over a longer period (~3 h).

2.2 Modified RUSITEC system

The classical RUSITEC system uses volumes between 500 and 1000 ml (Lengowski et al., 2016; Becker, 2012; Oetztuerk et al., 2005; Czerkawski and Breckenridge, 1977). For the purpose described herein, where the metabolism of radioactively labelled compounds was to be analyzed, the system was reduced to a smaller volume of 250 ml. Further, the connections within the system were made safer to correspond to radiosafety requirements (Fig. 1A,B). The tubing connecting the buffer reservoir with the fermenter was changed to medical supplies and luer-lock connections (ZVD-Messsystem; Sarstedt AG & Co. KG, Nümbrecht, Germany). This enabled affordable sterile and single use tubing. The original plastic tube for the buffer was replaced by a 25 cm long steel tube soldered to a luer-lock connection. This enabled an easy and safe daily change of the nylon feed bags without the risk of radioactive spillage (Fig. 1C). Six fermenters were placed in a water bath kept at 39.5°C by means of a thermostat (ED; JULABO GmbH, Seelbach, Germany) and monitored and documented with a temperature data logger (EBI 300; ebro Electronic GmbH, Ingolstadt, Germany) and an external sensor (TPC 300; ebro Electronic GmbH) (Fig. 1A). The simulation of stomach movement was achieved with a motor so that the buffer was pumped into the fermenter at a speed of 0.174 ml/min, leading to a total exchange of liquid phase in 24 h (ISM 931; ISMATEC Laboratoriumstechnik GmbH, Wertheim-Mondfeld, Germany). The overflow from each fermenter was collected into Nalgene bottles (Nalgene-HDPE-flask square 1 l; Thermo Fisher Scientific Inc., Waltham, MA, USA). Gas arising in the fermentation was not collected for this study but could escape through two 1 mm holes in the lids. A third hole was fitted with a pressure valve (Proportional Relief Valve SS-RL3S6MM; Swagelok Company, Solon, OH, USA) to avoid any leakage due to blockage of the tubing. The valve would open at pressures above 1 bar to release liquids and gas into a receptacle (UD 3 TUR-BAG 4 l; Sarstedt AG & Co. KG, Nümbrecht, Germany). Before the start of the experiments the fermenters were dismantled and cleaned by ultrasonication for 10 min (Elmasonic S30H; Elma Schmidbauer GmbH, Singen, Germany) in 5% deconex 20 NS-x (Borer Chemie AG, Zuchwil, Switzerland), washed in deionized water, air dried and fitted together.

2.3 Experimental design

RUSITEC buffer
NaCl 28 mM, KCl 7.69 mM, CaCl$_2$ 0.22 mM, MgCl$_2$ 0.63 mM, HCl 0.5 mM, NaH$_2$PO$_4$ 10 mM, Na$_2$HPO$_4$ 10 mM, NH$_4$Cl 5 mM, NaHCO$_3$ 97.9 mM, pH 7.35 (Becker, 2012; Oetztuerk et al., 2005).

Feed
To mimic regular feed intake, 2.5 g hay (*Dein Bestes – Alpen-Wiesenheu*; dm-drogerie markt GmbH & Co. KG, Karlsruhe, Germany) and 1 g pellets (sheep fodder S2; Raiffeisen-Kraftfutterwerk Mittelweser Heide GmbH, Hoya, Germany) were filled into nylon bags with 50 µm mesh (*In-Situ*-Bags 5 × 10 cm R510; ANKOM Technology, Macedon, NY, USA), closed and stored in Nalgene bottles at room temperate until use. Prior to filling, hay was ground for 7 s at 7000 U/min (Grindomix GM 200; RETSCH GmbH, Haan, Germany) into particles of different sizes. The pellets were ground in a mortar. This particle distribution was intended to mimic rumination and thus the content of the rumen in an intact animal.

Liquid and solid rumen content
The liquid rumen was filtered through a viscose cloth (Tork Long-Lasting Cleaning Cloth 90477; SCA GmbH, Ismaning, Germany). Then the pH and the redox potential of the filtrate was determined (see below) and 250 ml filled into each of four fermenters. The contents of two nylon bags with feed were filled into the fermenters. To one of these 7 g of solid rumen contents were added to inoculate with bacteria associated with solids. The fermenters were placed into the water bath and connected to the pump and buffer reservoir and the motor was set at three strokes/min to imitate peristaltic movement. The bag containing the solid rumen constituents was changed to a fresh feeder bag after 24 h, the second one after 48 h, such that each feeder bag was in the fermenter for 48 h and one was changed each day. Over the entire experimental period, pH and redox potential were determined in samples collected at the same time and stored at -20°C.
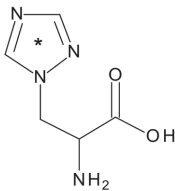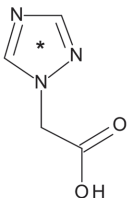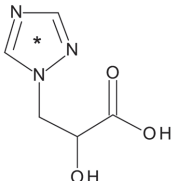
Incubations
To equilibrate the system, a pre-incubation for four or five days was performed during which pH, redox potential, particles and colony formation, protozoa and glucosidase activity were monitored (details see below). During this phase a stable population of microorganisms adapted to the *in vitro* conditions is achieved (Lengowski et al., 2016; Becker, 2012; Martínez et al., 2011). The amount of compound to be added was calculated under consideration of the efflux according to the following formula:

$$\frac{dS}{dt} = -\left(\frac{0.174}{250}\right)S$$

$$S(t) = S(0) * e^{-\left(\frac{0.174}{250}\right)t}$$

S: Amount of substrate in fermenter; t: time after addition; 0.174: efflux rate in ml; 250: volume of one fermenter in ml

The compounds used to examine the metabolism of the plant metabolites of azole-fungicides (Stroeher-Kolberg et al., 2014; FAO, 2007) were ^{14}C-labelled triazole-alanine (TA), triazole-acetic acid (TAA) and triazole-lactic acid (TLA) (Tab. 1). The test compounds were synthesized in the BASF SE isotope

Tab. 1: Reference compounds (TDM)

	Name	triazole-alanin (TA)
	Formula	$C_5H_8N_4O_2$
	Specific activity	0.02 MBq/mg
	Amount added	78.4 mg
	Dissolved in	2.5 ml VE-H_2O

	Name	triazole-acetic acid (TAA)
	Formula	$C_4H_5N_3O_2$
	Specific activity	0.02 MBq/mg
	Amount added	72.0 mg
	Dissolved in	2.5 ml VE-H_2O

	Name	Triazole-lactic-acid (TLA)
	Formula	$C_5H_7N_3O_3$
	Specific activity	13 MBq/mg
	Amount added	62.1 mg
	Dissolved in	6.2 ml VE-H_2O

(*) marks labelling with [14]C

laboratory and were labelled in the ring carbons of the triazole (formula specific activities and dosing see Tab. 1). The compounds were added each to one fermenter after the equilibration phase of 96 h. Samples of 2 ml were withdrawn after 4 h, 8 h, 24 h, 48 h, 72 h and 96 h, aliquoted into 2 Eppendorf tubes with 250 µl acetonitrile, vortexed (Reax 2000; Heidolph Instruments GmbH & Co.KG, Schwabach, Germany), and centrifuged for 5 min (Mikro 200; Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The supernatant was transferred to fresh Eppendorf tubes and stored at -20°C until analysis.

As model compounds for plant glycosides the ^{14}C-labelled octyl-β-D-glucopyranoside (American Radiolabeled Chemicals Inc., St. Louis, MO, USA) (unlabeled octyl-β-D-glucopyranoside was from Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and the unlabeled glycoside of resveratrol, polydatin (Sigma-Aldrich Chemie GmbH) were incubated in the modified RUSITEC system (Tab. 2). The compounds were added after 120 h equilibration. Rapid cleavage of octyl-glucoside by glycosidase was observed, therefore samples were taken at shorter intervals after 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 24 h, 48 h and 72 h.

2.4 Analytical methods

Determination of pH and redox potential

These were determined daily directly in the cultivated rumen fluid when the fermenter was opened to change feed bags. The pH was determined using a portable pH-meter (SG2 SevenGo; Mettler Toledo, Schwerzenbach, Switzerland) and a pH-electrode (InLab Expert Pro ISM; Mettler Toledo), the redox potential with a redox-electrode (LE 510; Mettler Toledo) after equilibration for 1 min.
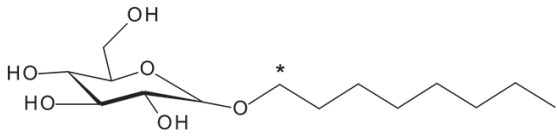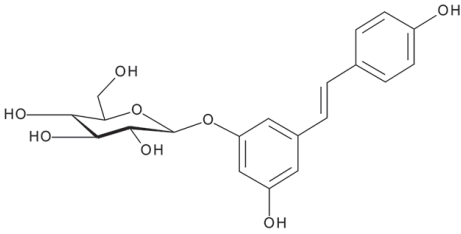
Viability tests

Samples of 1 ml were taken at the start of the fermentation, after equilibration (96 h) and at the end of the experiment after 168 h. Rumen was diluted 1:100, 1:1000, 1:10,000 and applied to agar plates (CASO-AbklatschA +LTH - RT+; heipha Dr. Müller GmbH, Eppelheim, Germany) and incubated for 24 h at 39°C (ED 23; BINDER GmbH, Tuttlingen, Germany) under anaerobic conditions (AnaeroGen W-Zip COMPACT; Oxoid Ltd, Basingstoke, UK). Colonies were counted after incubation (Modell 950 c; Fa. Schuett, Göttingen, Germany).

Determination of particles

Samples were analyzed every 24 h with an automatic Coulter counter (Moxi Z; ORFLO Technologies, Ketchum, ID, USA) in thin layer cassettes type S (MXC002; ORFLO Technologies) in the small particle mode. This setting measures particles of 2-10 µm diameter. Rumen contains particles between 0.4 and 10 µm diameter and some protozoa and flagellates have a diameter between 4 and 14 µm (Breves and Leonhard-Marek, 2010; Hungate, 1966). This method should therefore register any changes in the microbial composition of the rumen, taking into account

Tab. 2: Reference compounds (glycosides)

Name	Octyl β D glucopyranoside
Formula	$C_{14}H_{28}O_6$
Specific activity	0.07 MBq/mg
Amount added	108 mg
Dissolved in	2.5 ml RUSITEC-buffer

Name	Polydatin
Formula	$C_{20}H_{22}O_8$
Amount added	142 mg (Fermenter A)
	15 mg (Fermenter B)
Dissolved in	0.5 ml ethanol + 2 ml RUSITEC-buffer (Fermenter A)
	100 µl ethanol + 2.4 ml RUSITEC-buffer (Fermenter B)
	after sonication

that no distinction is possible between live cells and other particles. Protozoa were counted every 24 h in a Neubauer chamber directly in 10 µl rumen, recorded digitally (AxioCam MRc; Carl Zeiss Microscopy GmbH, Jena, Germany), and analyzed manually with the software AxiVision V. 4.9.1.0 (Carl Zeiss Microscopy GmbH).

Determination of β-glucosidase activity
β-Glucosidase activity in the samples stored at -20°C was determined by photometry at 405 nm (Synergy H1 Hybrid Reader; BioTek Instruments GmbH, Bad Friedrichshall, Germany) with 4-nitrophenol-β-D-glucopyranoside as substrate (β-Glucosidase Activity Assay Kit MAK129; Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Analysis of metabolites
Metabolites of the triazoles and glycosides were separated by HPLC and quantified by flow through scintillation or UV detection as follows:

The HPLC system for analysis of TA, TAA and TLA was composed of a Dionex Ultimate 3000 pump, a Dionex UCI-100 interface, a Dionex ASI-100 autosampler, and a Dionex STH 585 oven, steered by Gynkotek Chromeleon V6.80 SR5 software. For the ^{14}C-labelled compounds a radio-detector (Berthold LB507A) and a pump (Berthold LB5036) were used. Column for the triazole derivatives: TCI Dual ODS-CX15, 250 × 4.6 mm. Solvent A: $H_2O:CF_3COOH$ (1000:2.5) (v/v), solvent B: $H_2O:CH_3CN:CF_3COOH$ (5:995:2.5) (v/v/v), gradient: up to 15 min 100% A, 15 to 16 min 100% B, to 21 min 100% B, 22 min 100% A, flow 1 ml/min, oven temperature 24°C. Injection volumes were increased from 75 µl for samples withdrawn before 48 h to 100 µl for TA and TAA analyses, TLA samples from 50 µl at 4 h to 75 µl thereafter. Octyl-β-D-glucopyranoside and its metabolites were analyzed on the following system: Dionex P680 pump, Dionex UCI-50 interface, Dionex UltiMate 3000 autosampler, Berthold LB509 radiodetector and Berthold LB5035 pump, oven and software as above. The column was a Nucleodur C18 ISIS, 250 × 4.6 mm, solvent A: $H_2O:CH_3CN:HCOOH$ (950:50:1) (v/v/v), solvent B: $CH_3CN:HCOOH$ (1000:1) (v/v), gradient: up to 5 min 100% A, to 25 min 100% B, 30 min 100% B, 30.1 min 100% A. Flow 1 ml/min, temperature 25°C, injection volume 40 µl. Polydatin and metabolites (e.g., resveratrol) were analyzed according to Lv et al. (2006), with the same HPLC system and detected on a Jasco UV-2075Plus detector at 306 nm, the column was a Nucleodur C18 ISIS, 250 × 4.6 mm and the solvent $CH_3CN:H_2O:HCOOH$ (260:740:1) flow 1 ml/min, temperature 25°C, injection volume 20 µl of 1:2 diluted samples..

Statistics
Means of the fermenters and SD were calculated with Excel 2013 (Version 15.0.4763.1003; Microsoft Corporation, Redmond, WA, USA). The numbers and the sizes of particles were determined automatically; outliers were identified by Grubbs' test using the statistical program Minitab (Version 17.2.1; Minitab Inc., 16801 State College, PA, USA, significance levels α = 0.05). In accordance with the relevant OECD-regulations for studies on metabolism, a single HPLC determination was run.

3 Results

3.1 Vitality and stability of the RUSITEC system

The pH values determined every 24 h up to 192 h were stable between pH 6.52 and 6.83 with a mean of pH 6.70 (± 0.07 SD; n = 66). The redox potentials measured at the same time were also stable between -240 and -345 mV, with a mean of -301 mV (± 30 mV SD; n = 66). The colony forming assay to determine bacterial population showed an increase in colonies from 1.1 × 10E7 colonies/ml at the start of fermentation to 4.1 (± 0.8 SD) × 10E7 after 120 h to 4.8 (± 1.4 SD) × 10E7 after 192 h. The shape, color and structure of the colonies were unchanged as a function of time.

The concentration of particles in the liquid phase of the fermenter fell in the first 24 h from 178.6 × 10E5 particles/ml to 36.3 × 10E5 during the equilibration phase and then further

decreased to a stable value of $9.2 \times 10E5$ particles within the following timeframe up to 192 h (Fig. 2A). The size distribution also changed over the course of incubation (Fig. 2B). Generally, small particles were more abundant than larger particles. At the start of fermentation particles of 2-3.5 μm constituted 90%, after 24 h they constituted 70% of all particles (Fig. 2B).

The number of protozoa decreased over time from $63 \times 10E4/ml$ at time 0 to $1 \times 10E4$ at 120 h. The decrease was greatest within the first 24 h (Fig. 2C).

β-Glucosidase activity is expressed as units, where 1 unit cleaves 1 μmol 4-nitrophenol-β-D-glucoside in 1 min at pH 7.0. Glucosidase activity showed a drop corresponding to the drop in particles after 24 h incubation but then remained constant at 4.8 units/l until the end of the experiments (Fig. 2D).

3.2 Metabolism

The three derivatives of azole-fungicides showed distinctly different patterns of metabolism. Triazole-alanine (RT 5.4 min) was cleaved to 1.2.4-triazole (RT 6.8 min) after 24 h (Fig. 3B), leading to 67% of radioactivity as triazole after 96 h (Fig. 3D). Due to the dilution of the RUSITEC liquid phase by buffer over time, the absolute amount of substrate diminished and only relative distribution of radioactivity between the substrate and me-

tabolite could be calculated from peak areas. No further radioactive metabolite was detected. In contrast, triazole-acetic acid (TAA) and triazole-lactic (TLA) acid were stable in the modified RUSITEC system up to 48 or 96 h. No radioactive peak except the parent compound TAA with a retention time (RT) of 4.7 min (Fig. 3E-F) and of TLA with a retention time of 6.3 min were detectable (Fig. 3G-H).

Cleavage of octyl-β-D-glucopyranoside was very effective in forming an octanol derivative already detectable after 5 min of incubation (Fig. 4A), leading to complete degradation after 30 min (Fig. 4D). At later incubation time points up to 24 h an unknown metabolite with a 1 min shorter retention time than the octanol-derivative was observed (data not shown).

The resveratrol glucoside polydatin was incubated at two different concentrations. When only 15 mg were added to the fermenter, polydatin was cleaved to resveratrol by β-glucosidase in 5 min (Fig. 4E), whereas after adding 142 mg polydatin a slower cleavage over 1 h was observed (Fig. 4F). The metabolite resveratrol seemed stable over 6 h since no further peak with an absorption at 306 nm was observed. After 24 h no resveratrol was measurable. Here, as in incubations with the labelled compounds, no quantitative measurements were possible due to the dilution of the rumen fluid with buffer over time.
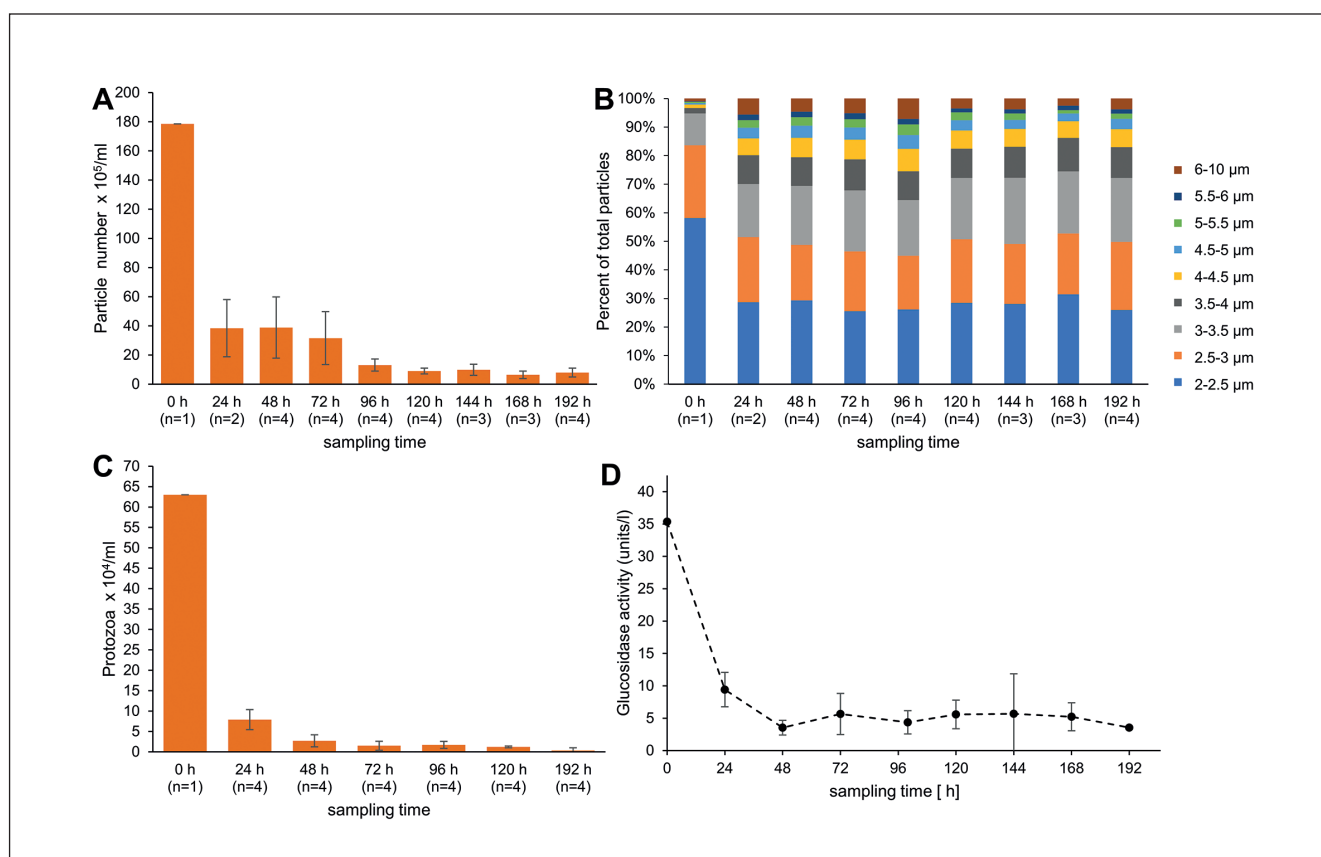

Fig. 2: Results on the microflora and its activity in the fermenter over time
A, Particle density ($\bar{x} \pm SD$); B, Distribution of particles according to size; C, number of protozoa in the liquid phase ($\bar{x} \pm SD$);
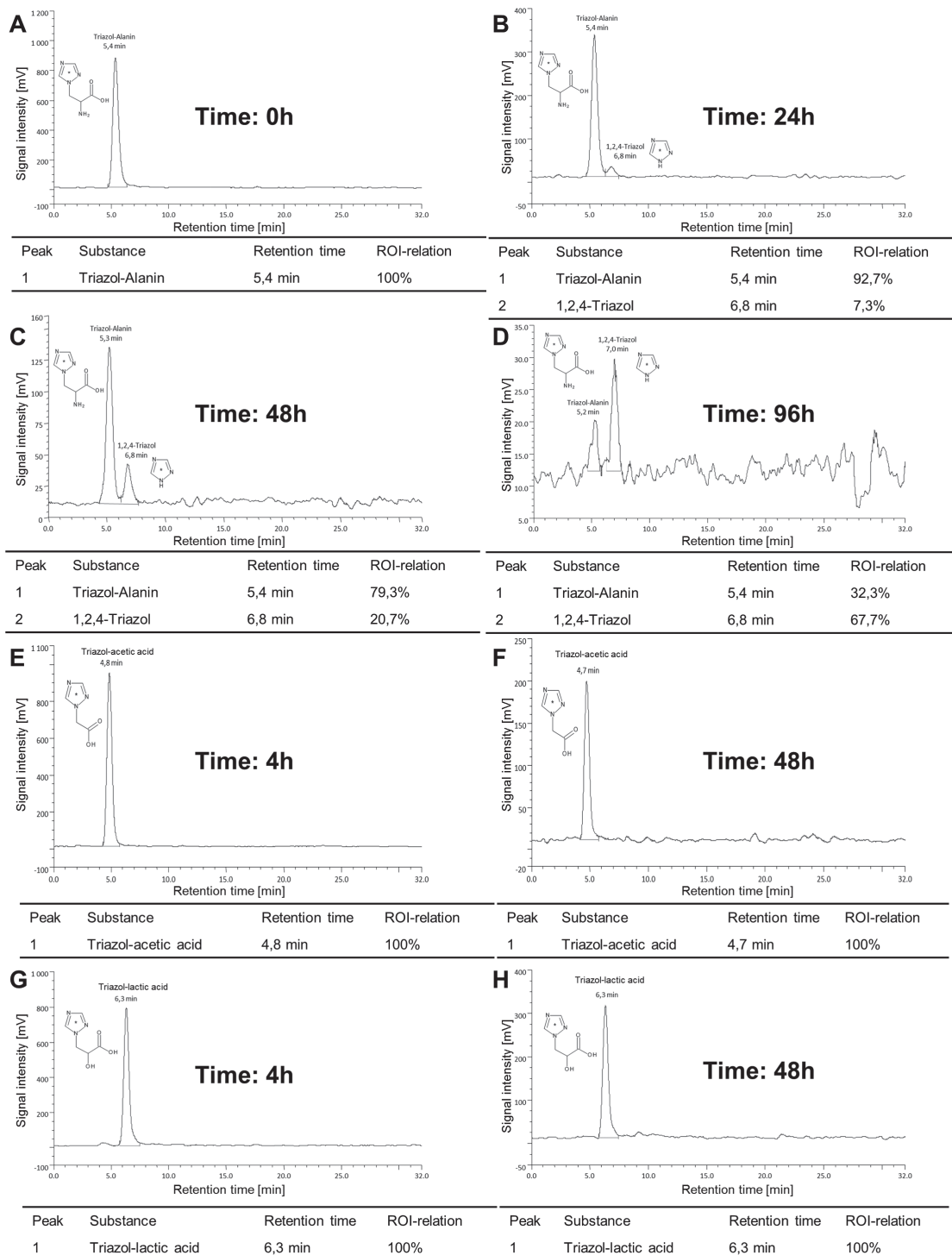D, Mean β-glucosidase ($\bar{x} \pm SD$), n = 4.

Fig. 3: Incubation of different triazole derivative metabolites and formation of 1,2,4, triazole (B-D) and stability (E-H) at different timepoints

A-D, Incubation of ^{14}C-triazole-alanine at the start of the incubation (A), after 24 h (B), after 48 h (C), and after 96 h (D); E-F, Incubation of ^{14}C-triazole-acetic-acid after 4 h of incubation (E) and after 48 h (F); G-H, Incubation of ^{14}C-triazole-lactic-acid at 4 h of incubation (G) and after 48 h (H).
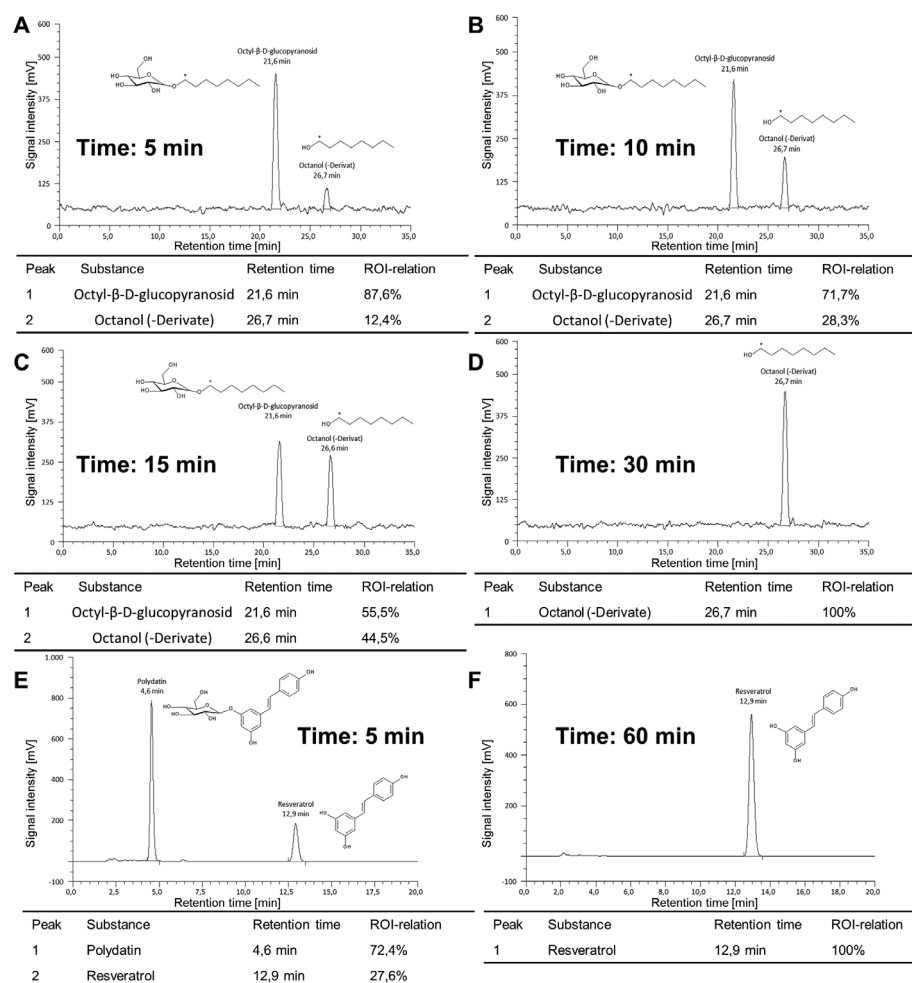
Fig. 4: Incubation of glycosides and the degradation of ¹⁴C-octyl-β-D-glucopyranoside (A-D) or polydatin (E-F) at different timepoints

A-D, Incubation of ¹⁴C-octyl-β-D-glucopyranoside for 5 min (A), after 10 min (B), after 15 min (C), and 30 min (D). E-F, Incubation of polydatin for 5 min (E) and 60 min (F).

4 Discussion

The modified RUSITEC system was developed to facilitate incubations with radioactive compounds. The key features are a smaller volume, safe luer-lock connections and single use affordable sterile tubing. Because the conditions differ from those of the original RUSITEC system developed by Czerkawski and Breckenridge (1977), vital parameters such as pH, redox potential and microbial populations were analyzed. The pH was as described in the literature and remained stable at pH 6.70, which is in the range of 5.0 to 7.5 recommended by Czerkawski (1986). The redox potentials equally remained stable during the experiment between -240 and -345 mV. Different values are given in the literature for normal redox potentials in rumen. Breves and Leonhard-Marek (2010) determined values between

-250 and -300 mV, while Barry et al. (1977) determined potentials in sheep of -150 to -260 mV and Marounek et al. (1982) found values between -145 and -190 mV in goats. Marden et al. (2005) describe these discrepancies in detail and explain them by using different reference electrodes with and without corrections. A study using the same electrode as we did obtained values in the same range as measured here (-170 to -300 mV; Becker, 2012). Therefore, it appears that the anaerobic conditions necessary for rumen metabolism were stable during the experimental procedures.

The determination of bacterial colonies showed an increase in colonies in the first 24 h followed by a stable population over time. This is in accordance with studies with a comparable approach of measurement (Hungate, 1966; Kistner, 1960). But there are also values in the literature that show higher numbers

of 10E10 to 10E11 cells/ml fresh rumen. The reason for the much lower numbers determined after plating in culture like in this approach is that many of the rumen bacteria do not grow on standardized culture plates (Hungate, 1966). As with other enterobacteria, the culture conditions for rumen bacteria are not yet optimized to enable stable growth *in vitro* (Breves and Leonhard-Marek, 2010). Molecular biology allows analysis of these bacteria (Wang et al., 2015) and a recent publication (Lengowski et al., 2016) has shown the dynamics of several rumen microorganisms in the RUSITEC system. These authors showed a decrease in most species in the first 24 h of cultivation, leading to a stable population at later incubation times. The loss of protozoa was most drastic with a factor of 10-50. This was also observed in our modified RUSITEC system, where protozoa decreased from $6.3 \times 10E5$ protozoa to $1 \times 10E4$ protozoa per ml rumen. The physiological range is wide between 10E5 to 10E6 protozoa per ml rumen (Hungate, 1966). Others also have described the loss of protozoa in the RUSITEC system (Lengowski et al., 2016; Becker, 2012; Martínez et al., 2011; Ziemer et al., 2000). The reason discussed for these observations is the loss due to buffer efflux, their long generation time of two to three days (Fuchigami et al., 1989), or adherence of protozoa to feed particles in the nylon bags (Czerkawski and Breckenridge, 1979), which are thus not accessible in the sampled liquid. There are, however, indications that loss of protozoa does not significantly influence metabolism in the rumen (Breves and Leonhard-Marek, 2010).

The number of particles determined by Coulter counting also diminished drastically in the first 24 h. Here again the flow of buffer surely dilutes particles. In addition, the automatic system does not differentiate between inert particles and microorganisms. A stable value was reached after 24 h. The size distribution also stabilized with particles between 2 and 3.5 μm being most abundant. According to Hungate (1966), most of them are 0.4-1.0 μm wide and 1-3 μm long. Larger bacteria and smaller protozoa such as flagellates, which are 4 to 14 μm in size (Breves and Leonhard-Marek, 2010), will therefore constitute most of the counted microorganisms. Our data cannot fully be compared with others, nevertheless the obtained data can be used to check changes in the fermentation conditions and be used as an internal quality check. The size distribution with the smallest particles comprising 70% of all particulate matter suggests that these were indeed live cells that attained a stable population during our experiments.

The β-glucosidase activity profile closely follows that of the particles in the rumen and reaches a stable value after 48 h of 4.8 (± 1.0 SD) units/l. Values in the literature are much higher but cannot be directly compared, since assay conditions as well as unit definitions are different. Agarwal et al. (2002) and Wang et al. (2015) defined 1 unit as the cleavage of 1.0 μmol substrate at pH 4.5 and 70°C. In addition, these authors lysed cells in the rumen by vortexing and a freeze-thaw cycle, thereby liberating microbial β-glucosidase.

Metabolism studies of triazole derivative metabolites like triazole-alanine (TA), triazole- acetic-acid (TAA) and triazole-lactic-acid (TLA) (Stroeher-Kolberg et al., 2014; FAO, 2007)

in the modified RUSITEC system are important contributions to the investigations requested by EFSA for plant metabolites of azole-fungicides (EFSA, 2013). Our studies show that triazole-alanine (TA) is metabolized to 1,2,4-triazole and that after 96 h half of the added TA is cleaved. The amounts incubated are however larger compared to the amounts expected to be ingested by ruminants *in vivo*. Analysis of TA in fodder resulted in values of 0.1 mg TA/kg wet weight (Stroeher-Kolberg, 2014). Ruminants would therefore eat less than 1 mg/kg fodder. Sheep, which eat 1-2 kg feed per day, would ingest a maximum of 2 mg TA into 5 l of rumen (Orpin and Letcher, 1984). In our *in vitro* experiment, this would amount to 0.1 mg TA per fermenter of 250 ml, which would then likely be rapidly metabolized to triazole. The other two test compounds triazole-acetic acid (TAA) and triazole-lactic acid (TLA) were completely stable and were not degraded (Fig. 3). The selective metabolism, namely cleavage of TA and stability of TAA has been shown *in vivo* in an OECD 505 study in cows (TDMDG, 2016). This shows clearly the predictivity of the RUSITEC method. No data on ruminant metabolism of TLA are currently available, neither OECD 501 nor OECD 505 studies. But, since data are requested by authorities, the data of this publication have been submitted to the British Chemicals Regulation Directorate (CRD) (TDMDG, 2016). If approved, a feeding study with 30 cows according to OECD TG 505 could be avoided.

Glycosides are very common in plants (Jenner et al., 2005; Bennett and Wallsgrove, 1994) and can be formed by intrinsic plant constituents but also from exogenous compounds such as plant protection active ingredients or their metabolites. During the approval process of plant protection products, the fate of such glycosides, ingested by ruminants, must be determined. The metabolism of octyl-β-D-glucopyranoside as model compound and polydatin, the glucoside of the plant constituent resveratrol, was analyzed. Since β-glucosidase activity in the modified RUSITEC system was stable after equilibration, an effective cleavage of both compounds was expected. This was indeed the case: octyl-β-D-glucoside was cleaved after 5 min to a metabolite with lower polarity than the glucoside (Fig. 4A). Unequivocal identification of this metabolite as octanol was not possible. It is therefore denoted as octanol(-derivative) in the figure. After 30 min, the parent compound had disappeared completely and only octanol or its derivative remained. Here the amount of substrate added to the fermenter was also high to enable detection after the dilution steps caused by exchange of buffer. At physiological concentrations of a glucoside a very rapid cleavage to the aglycon can be expected.

Also, polydatin was rapidly cleaved and resveratrol was detectable after 5 min incubation of 142 mg polydatin. After 1 h only resveratrol was detectable (Fig. 4E,F). When more physiological concentrations of 15 mg polydatin were fermented, only resveratrol was measurable after 5 min, showing a rapid metabolism of this glucoside under physiological conditions. Resveratrol itself seemed stable for up to 6 h since no further metabolite detectable at 306 nm was visible, but after 24 h no resveratrol was detectable, leading to the assumption that resveratrol is indeed further metabolized in the RUSITEC

system. The loss of resveratrol is higher than expected by dilution of the liquid phase. Since no radioactive polydatin was available, any further metabolism could not be investigated. Nevertheless, these data show that the system has a very high metabolic capacity and seems to mimic the *in vivo* situation of ruminants well.

5 Conclusions

The regulatory agencies often require further animal studies in ruminants to investigate the fate and therefore further xenobiotic metabolism of metabolites of applied active ingredients to conduct a data-based risk assessment for pesticides (EFSA, 2015). The modified RUSITEC system presented herein can replace these additional requested xenobiotic metabolism studies in livestock for the registration process of plant protection products or veterinary drugs. The system maintains the particular properties of rumen and, after a pre-incubation time, is stable for up to 192 h. The modification described herein has not changed the metabolic capacity described over many years for the conventional 500-1000 ml RUSITEC system. The simplification and safer handling of the modified RUSITEC makes it possible to use the system in routine applications and to handle both radiolabeled and unlabeled compounds. Since the selectivity of the metabolism was proven by *in vivo* studies, BASF will replace *in vivo* animal studies on ruminant metabolism studies beyond OECD 503 by performing RUSITEC studies. The method is already included in the common method portfolio of BASF, leading to a significant contribution to the 3R strategy. Further studies comparing data with *in vivo* observations will show the usefulness of the system and will in future prevent many animal studies and provide alternatives to animal experiments in the sense of the 3Rs.

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Conflict of interest

The authors declare no conflicts of interest.