Predicting the Transfer of Contaminants in Ruminants by Models – Potentials and Challenges

Fenja Klevenhusen¹, Karl-Heinz Südekum², Gerhard Breves³, Franziska Kolrep¹, Manfred Kietzmann¹, Pietro Gerlettì, Jorge Numata¹, Markus Spolders¹, Robert Pieper¹ and Janine Kowalczyk¹

¹German Federal Institute for Risk Assessment, Berlin, Germany; ²Institute of Animal Science, University of Bonn, Bonn, Germany; ³Institute for Physiology and Cell Biology, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany; ⁴Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

Abstract

Certain undesirable substances in feed can transfer into foods of animal origin after ingestion by livestock animals. They become contaminants in food that may threaten the consumers’ health. Commonly, feeding trials with animals are conducted to assess the transfer of undesirable substances into animal tissues or milk. Such feeding trials explore the effects on transfer of the various physiological systems (e.g. ruminant and non-ruminant gastrointestinal tracts) as well as different livestock production intensities. Using alternative methods to mimic the complex physiological processes of several organs is highly challenging. This review proposes a potential cascade of in vitro and ex vivo models to investigate the transfer of contaminants from feed into foods of animal origin. One distinct challenge regarding the models for ruminants is the simulation of the forestomach system, with the rumen as anaerobic fermentation chamber and its epithelial surfaces for absorption. Therefore, emphasis was given to in vitro systems simulating the rumen with its microbial ecosystem as well as ex vivo systems to replicate epithelial absorption. Ruminants contribute significantly to the food sector, not only through meat but also through milk production. The transfer from blood into milk has to be evaluated by employing a suitable model. At the end, in silico approaches are introduced that can fill the gaps or substitute in vitro and ex vivo models. The integrative in silico method of physiologically-based toxicokinetics puts together the information gained from all alternative methods to simulate the transfer of ingested undesirable substances into foods of animal origin.

1 Introduction

Expectations of animal-derived food are high. Besides being available in sufficient quantities, animal-derived food is expected to be of high nutritional value, and, most importantly, food safety risks for the consumers’ health have to be low. Undesirable substances in feed can pose a threat to the consumer’s health if, upon ingestion by livestock animals, they are transferred into foods of animal origin, where they are further considered as contaminants. ‘Undesirable substances’ in EU legislation on animal nutrition mean any substance or product, with the exception of pathogenic agents, present in and/or on the product intended for animal feed that presents a potential danger to human health, animal health or the environment or adversely affects livestock production (Directive 2002/32/EC). In comparison, contaminants are substances that have not been intentionally added to but may be present in food as a result of the various stages of its production, packaging, transport or storage. They also might result from environmental contamination (Council regulation (EEC) No 315/93). Accordingly, undesirable substances are regulated for the contents in feed, while contaminants are regulated for their contents in food. REACH (Regulation (EC) No 1907/2006) requires, according to Article 14(4), exposure assessment and subsequent risk characterization to be carried out for chemical substances subject to registration that are manufactured or imported in quantities equal to or greater than 10 (metric) tons per year, and where the substance fulfils the criteria for any of the hazard classes or categories indicated in Article 14(4) or is assessed to be persistent, bioaccumulative and toxic (PBT) or very persistent and very bioaccumulative (vPvB). As such, for substances in plant protection products (Council Directive 91/414/EEC) as well as for the authorization of several feed additives (Commission Regulation (EC) No 429/2008), investigations on a possible transfer from feed to food are mandatory to establish withdrawal periods, maximum residue levels (MRLs) or maximum contents in feedingstuffs (Directive 2002/32/EC). This routinely requires feeding trials with various livestock species to cover the different physiological systems (e.g. ruminant gastro-intestinal tract (GIT) and non-ruminant GIT). These in vivo trials are time-consuming, expensive and often require the killing of the involved animals to...
harvest organs and tissues for contaminant analyses (e.g. according to OECD guidelines for testing of chemicals OECD 503 and OECD 505; OECD 2007a,b). Following the 3R principle (Replace, Reduce, Refine) by Russel and Burch (1959), research is carried out to develop alternative models (in vitro, ex vivo, in silico) to simulate the relevant in vivo processes as closely as possible. In order to employ them for regulatory purposes, their development needs to undergo a thorough validation step to ensure scientific integrity and quality (OECD, 2018).

Various partial gastro-intestinal in vitro and ex vivo systems have already been established decades ago to evaluate the nutritional value of feedstuffs. As such, they can simulate the process of nutrient digestion and absorption in the GIT. However, so far they have only rarely been used to study the transfer and metabolism of undesirable substances in the animal. Moreover, to investigate a potential substance transfer into animal tissues or excreta when ingested with feed, the physiological processes downstream need to be simulated by a cascade of individual methods to understand the whole complexity of animal metabolism. Simulating processes in the ruminant animal is especially challenging due to the physiological distinctiveness of the forestomach system and the animal’s highly different metabolic statuses in life, as e.g. dairy cows during the onset of lactation vs. beef cattle.

Recently introduced in silico models may help to simulate the potential interactions between individual body compartments and simulate the species-specific differences. The decision on whether non-animal methods are suitable for reproducing the metabolism of an undesirable substance in the animal requires a detailed examination of the functioning, potential and limitations of each individual method (Tab. 1).

The present report reviews established in vitro and ex vivo models to simulate the physiological processes in ruminant species, beginning with the complex forestomach system, including intestinal absorption and hepatic metabolization and ending in the excretion via milk. The focus is on the fate of foreign substances in the organism. In this context, the fate is called kinetics and the substances xenobiotics. In the end an in silico approach of kinetic modelling is introduced to model the interactions between the individual in vitro and ex vivo compartments to complete the understanding of the entire animal metabolism and close the gap between in vitro and in vivo results.

2 **In vitro reticulo-ruminal digestion and fermentation**

2.1. **General considerations regarding ruminant species**

Ruminants are unique in their mode of plant digestion, as they have evolved a forestomach, which allows microbial digestion and fermentation of the fibrous carbohydrates cellulose, hemicelluloses leading to the formation of short chain fatty acids (SCFA), which constitute the major energy source for the ruminant animal (Bergman, 1990). The ruminant’s stomach system is composed of the four parts reticulum, rumen, omasum, and abomasum. The reticulum and rumen are joined by a fold of tissue and constitute a large fermentation chamber that houses a highly diverse rumen microbiome with manifold known enzymatic activities (Morais and Mizrahi, 2019). The rumen wall is covered by a keratinizing stratified squamous epithelium in the form of countless papillae which is effectively absorbing and secreting. In addition, it constitutes an effective permeation barrier (Aschenbach et al., 2019). The reticulum is further connected to the omasum, which is also called manyplies because of the numerous parallel sheets of tissue and serves as bottleneck for feed particle passage from the reticulo-rumen. Fifteen percent of the water that enters the omasum are absorbed here (Krehbiel, 2014). The following abomasum is similar to the non-ruminant stomach containing a glandular gastric mucosa with specialized secretory cells that produce mucus, pepsinogen, and hydrochloric acid.

For decades experiments have been conducted to compare ruminant species for their intake and digestive capacity by feeding a wide range of rations with different ingredients and chemical composition. In a meta-analysis study, Riaz et al. (2014) stated that numerous studies that have compared feed intake and nutrient digestibility between sheep and goats and between sheep and cattle and few studies have made comparisons between cattle and buffaloes and even less studies have been published on the comparison of feed intake and digestibility among more than two ruminant species. From these comparative studies it appears that differences exist between ruminant species, both in intake and digestive capacity at the reticulo-ruminal and total digestive tract level, and additional differences between genotypes, e.g. between Jersey versus Holstein-Friesian cows, have to be considered (Beecher et al., 2014).

However, others have observed that no systematic differences occurred between sheep and cattle in digesting maize silage and grassland products (Aerts et al., 1984). Moreover, interspecies differences in many studies were, in part, related to differences in passage rates of solids (Bartocci et al., 1997) or fluid (Colucci et al., 1990), or both (Francoise Domingue et al., 1991; Popp et al., 1980), from the reticulo-rumen. It may therefore be argued that reticulo-ruminal in vitro systems, where reticulo-ruminal retention times or the reciprocals, i.e. outflow rates are constantly controlled regardless of ruminant species, are less prone to be affected by species differences than in vivo studies. Therefore, the following considerations will focus on studies which compared ruminal contents from different species in in vitro experiments.

More than 30 years ago, Kudo et al. (1984) have shown that the toxic amino acid mimosine was degraded to a similar extent in vitro in ruminal fluid from both cattle and sheep and that the degradation occurred faster when the donor animals were fed a mixed forage-concentrate ration than forage alone. When grass silage or maize silage were incubated in a rumen simulation technique (RUSITEC) system using ruminal contents from either cows or sheep fed on three different diets, Boguhn et al. (2013) observed that in vitro nutrient degradation and microbial crude protein synthesis were more affected by the ration fed to the donor animals than the animal species and was probably mediated by an adjusted microbial community. This was confirmed by Witzig et al. (2015) who analyzed the composition of the microbial community in the RUSITEC experiment of Boguhn et al. (2013) and found that the effect of donor animal species was limited to the number of archaea, which was greater for sheep than for cows. These authors concluded that the rumen microbial community that establishes in vitro is primarily affected by the ration of the donor animals. Based on their findings, the authors suggested using a standardized approach for studying the rumen microbiota in a rumen simulation. This suggestion was underlined in a recent
study (Belanche et al., 2019), in which the authors emphasized that collection time, donor animal diet, fermentation substrate and inoculum preservation method may all have an impact on the variables that are being studied.

In general accord with the observations outlined above, Henderson et al. (2015) reported that differences in ruminal microbial community compositions were predominantly caused by ration and much less by the host. Moreover, a core microbiome was found across geographical regions and ruminant species, such that similar bacteria and archaea dominated in nearly all samples, and only protozoal communities were more variable. Accordingly, the “core bacterial microbiome” at the genus or higher levels comprise Prevotella, Butyrivibrio, and Ruminococcus, as well as unclassified Lachnospiraceae, Ruminococcaceae, Bacteroidales, and Clostridiales, providing a multitude of enzymes needed for the breakdown of nutrients (Hartinger et al., 2018; Huws et al., 2018; Moraïs and Mizrahi, 2019).

<table>
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<tr>
<th>Technique</th>
<th>Simulated or replaced organ or tissue</th>
<th>Pros</th>
<th>Cons</th>
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<tr>
<td>Continuous culture models</td>
<td>Rumen, colon, caecum</td>
<td>- No killing of animals needed</td>
<td>- Donor animals needed; one-time surgery required</td>
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<td>- Long term incubation allows investigation of bacterial adaptation to xenobiotics</td>
<td>- Cannulated animals need extra care</td>
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<td>- Allows simultaneous investigation of several substances or dosages</td>
<td>- Loss of ruminal protozoa</td>
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<td>- No epithelial absorption and secretion</td>
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<td>- Time intensive</td>
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<td>- Apparatus commonly not commercially available</td>
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<tr>
<td>Batch culture</td>
<td>Rumen, colon, caecum</td>
<td>- No killing of animals needed</td>
<td>- Donor animals needed; one-time surgery required</td>
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<td></td>
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<td>- Easy and fast</td>
<td>- Cannulated animals need extra care</td>
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<td>- Allows simultaneous investigation of several substances or dosages</td>
<td>- No epithelial absorption and secretion</td>
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<td>- Short term incubation likely does not allow microbial adaptation to inoculation conditions and xenobiotics</td>
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<td>Ussing chamber</td>
<td>All gut segments</td>
<td>- Different gut segments can be tested simultaneously</td>
<td>- Animals have to be killed</td>
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<td>- Viability of tissues is limited</td>
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<td>- Costly and laborious</td>
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<td>Immortal epithelial cell lines</td>
<td>Rumen, small intestine</td>
<td>- No killing of animals needed</td>
<td>- Sometimes different physiological behavior compared to in vivo conditions</td>
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<td>- Rapid screening of various contaminants, their concentration and interaction</td>
<td>- No/limited interaction with other cell types (e.g. immune cells)</td>
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<td>- Allows studying mechanisms at cellular level</td>
<td>- Missing mucus layer</td>
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<td>- Results are valid on cell level but cannot necessarily be extrapolated to complex in vivo conditions</td>
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<td>Liver S9 mix and microsomes</td>
<td>Liver, intestine, kidney, lung, skin</td>
<td>- Reduced number of animals as organ donors needed</td>
<td>- Microsomes contain only membrane-bound enzymes</td>
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<td>- High ethical acceptance</td>
<td>- Addition of cofactors required</td>
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<td>- Cheap and easy to produce</td>
<td>- Investigation of transport mechanisms not possible</td>
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<td>- S9 contains both membrane-bound and cytosolic liver enzymes</td>
<td>- Comparability to the in vivo situation is lower than for immortalized and primary cell lines</td>
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<td>- Phase I and phase II reactions can be specifically investigated by the addition of appropriate cofactors</td>
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<td>- Available from all relevant metabolizing organs</td>
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<td>Organ perfusion models</td>
<td>Liver, kidney, udder</td>
<td>- Complex organ cellular interaction</td>
<td>- Animals have to be killed</td>
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<td>- Active in vivo-like transport and metabolism conditions</td>
<td>- Viability of tissues is limited</td>
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<td>- Better understanding of quantitative contribution of the organ to certain processes</td>
<td>- Ischemia/reperfusion-caused cellular stress/damage</td>
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<td>Toxicokinetic modelling</td>
<td>All organs and tissues</td>
<td>- No killing of animals needed</td>
<td>- Reliance on extensive datasets</td>
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<td>- Can generate predictions on future scenarios</td>
<td>- Some data needs to be generated by animal experiments (for now)</td>
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<td>- Extrapolation to different species, chemicals and settings possible</td>
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<td>- Cheaper than animal experiments</td>
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In summary it appears that, for in vitro studies on rumen metabolism, ruminal contents from different ruminant species are equally suitable, provided that feeding management of animals and handling of ruminal fluid is standardized and procedures are applied consistently regardless of origin of ruminal fluid.

2.2. Continuous culture models

The rumen harbors a complex anaerobic ecosystem consisting of different niches of microbes, which enable extensive degradation and fermentation of feedstuffs. Diverse microbes have been shown to either adhere to fibre particles, or being rather associated with the liquid phase (McAllister et al., 1994; Klevenhusen et al., 2017) or attached to the rumen epithelium (Petri et al., 2013; Wetzel et al., 2016). Accordingly, in vitro systems simulating the rumen fermentation have to ideally preserve this complex microbial community mirroring the original rumen microbiota and fermentation processes and inoculation of the in vitro fermenters with the original rumen microbiota from donor animals is necessary. Ruminal contents for inoculation can be harvested either from slaughterhouses or from ruminally cannulated animals. The advantages of using ruminally cannulated animals are that their medical history is known and their feeding management can be controlled and adapted to specific research questions. Commonly the cannulated animals are kept in research facilities with close access to the laboratories for in vitro incubation. This ensures short transport ways necessary for microbial survival. Contrastingly, it is often difficult to find slaughterhouses in close proximity to research facilities and transport ways are too long to maintain the ruminal microbiota. Although ruminally cannulated animals can live long, healthy lives, the cannulation itself is a surgical intervention considered as animal experiment and thus requires sound justification.

Several continuous culture systems have been developed in the 1960s (Aafjes and Nijhof, 1967), however, applicability was often limited. Finally, there are basically three major systems, which are routinely used in laboratories worldwide. One is the rumen simulation technique (RUSITEC), which has been established in the 1970s by Czerkawski and Breckenridge (1977 and 1979a, b). The second one is the system first described by Slyter et al. (1964), in which solid feed is added directly to the fermenters and the overflow is collected. The third one is the dual flow continuous culture system designed by Hoover et al. (1976), in which solid feed is also added directly to the fermenters, but the overflow of liquid phase and the solid phase leave the fermenters separately at different outflow rates.

The RUSITEC system typically comprises 4 to 8 fermenters, each having a volume of 700 to 1000 ml. Artificial saliva (e.g., McDougall, 1948) is infused continuously at the bottom of the vessel and the overflow of the liquid phase and the fermentation gases are forced through an outflow by a small positive pressure in the gas space. The overflow and the produced gas can be collected quantitatively in bottles and gastight bags, respectively, for quantification and analysis. Degradability of the diets is commonly determined from synthetic fibre feed bags after 48 h of incubation. Pore sizes of the feed bags vary between studies, from 50 to 200 μm. On the first day of a RUSITEC experiment each fermenter is filled with a mixture of rumen fluid from at least three donor animals as proposed by Udén et al. (2012) and artificial saliva. In addition, approximately 80 g solid material of the rumen fiber mat (Czerkawski and Breckenridge, 1977) is provided in a feed bag to enable the establishment of both, liquid and particle associated ruminal microbes. An additional feed bag contains the experimental diet to ensure nutrient delivery to the microbes. Both bags are placed into the “food container” inside of each fermenter, which is continuously moved up and down to warrant constant mixing of the fluid and the solid phase (Czerkawski and Breckenridge, 1977). Feed bags are usually inoculated in the fermenters for 48 h before being replaced by fresh ones. In routine RUSITEC experimental runs, the digestibility of different feedstuffs or diets, as well as fermentation variables such as the production of short chain fatty acids (SCFA) and methane can be determined in a relatively short time of 10 to 15 days (e.g. Hindrichsen et al., 2004; Khaosa-Ard et al., 2009; Terry et al., 2018), however, also longer experiments of more than 20 days have been successfully conducted (e.g. Wallace and Newbold, 1991; Soliva et al., 2004). To obtain statistically validated results, though, sufficient numbers of replicates need to be obtained by repeating the experimental runs, thus prolonging the whole experiment to several weeks.

One limitation of the RUSITEC is that, although bacterial populations can be largely maintained for several days and even weeks (Ziemer et al., 2000; Wetzel et al., 2018), protozoal populations have been shown to decrease considerably in abundance already after few days (Ziemer et al., 2000; Martínez et al., 2010b; Lengowski et al., 2016). This is likely a result of the high liquid turnover rate in comparison to the generation intervals of the protozoa (Potter and Dehority, 1973). Single and dual flow continuous culture systems as proposed by Slyter et al. (1964) and Hoover et al. (1976) on the other hand have been shown to maintain also the protozoal populations (Miettinen and Setälä, 1989), albeit this strongly depends on turnover rate and stirring conditions applied (Mansfield et al., 1995; Moumen et al., 2009). Hoover et al. (1976) designed a dual effluent removal system to simulate the differential flows for liquids and solids found in the rumen allowing a longer residence time for the digestion of solid particles. A variable rate mechanical feeding device apportions the feed into the fermenter in regular intervals during the day. A magnetic stirrer operates intermittently, stirring the contents for 5 min every 5 h at low speed to minimize possible deleterious effects of mechanical agitation on the protozoa. However, in comparison to the RUSITEC system, which is commonly inoculated with ruminal fluid and solid material from the ruminal fiber mat, the systems of Slyter et al. (1964) and Hoover et al. (1976) are inoculated only with ruminal liquid. Thus, although protozoa might survive for a longer period and in greater abundance, ruminal microbes attached to the fiber mat might be generally missing or are at least underrepresented.

Continuous culture approaches are often used to investigate dietary effects and effects of feed additives on fermentation characteristics, such as nutrient degradation and methane formation (e.g. Soliva et al., 2004; Busquet et al., 2005; Terry et al., 2018; Petri et al., 2019). However, so far only few studies have used any of the described continuous culture techniques to investigate the ruminal degradation or biotransformation of undesirable substances.

For instance, researchers analysed the effects of Fusarium toxins on nutrient utilization, the turnover of deoxynivalenol and zearalenone (Seeling et al., 2006) and the responses of the rumen microbial communities to Fusarium-contaminated feed (Strobel et al., 2008; Bojahn et al., 2010) within RUSITEC. In their studies they could show that deoxynivalenol and zearalenone were only slightly metabolized by the in vitro rumen microbiota. Koch et al. (2006)
demonstrated alterations in the microbial population structures and their metabolic profiles in the presence of transgenic maize, while application of a glyphosate-containing herbicide in concentrations reflecting potential exposure of dairy cows or beef cattle did not exhibit significant effects on bacterial communities in RUSITEC (Riede et al., 2016). Kowalczyk et al. (2015) studied the recovery of perfluorovinyl acids during the incubation of contaminated feed in RUSITEC. Recently, Birk et al. (2018) modified the RUSITEC system in way that allowed testing 14C-labelled metabolites of azole fungicide and thus proved that the system can also be used to work with radiolabeled test compounds.

One reason for the lack of more studies might be the relatively long duration of a complete experiment to achieve a reasonable number of replicates for the statistical evaluation. Other reasons might be the change in microbial populations and community structure with prolonged time of incubation and the constant dilution by buffer infusion, which has to be taken into account. Accordingly, short term batch culture approaches might be an easier to conduct and more appropriate method to investigate microbial effects on the fate of contaminants in ruminal fluid (see paragraph 2.3.).

Although the continuous culture approaches can maintain a steady fermentation pattern, which is very similar to the pattern observed in the rumen of the donor animals (Hannah et al., 1986; Mansfield et al., 1995; Martinez et al., 2010a), over several days up to weeks, it remains to be answered whether the in vitro degradation and biotransformation of contaminants from feeds fully represent in vivo microbial processes, as the epimural microbiota, which is associated with the ruminal epithelium, is completely missing in vitro, and the fiber-attached microbes (i.e. bacteria and fungi) and protozoa are underrepresented. Nevertheless, the continuous culture approaches can provide information on whether certain contaminants are likely to be transformed by ruminal microbes or not.

Besides the epimural microbiota, it should be stressed that so far none of the developed incubation systems includes the simulation of the ruminal epithelium with its capacity for absorption of fermentation acids and possible harmful substances as well as its release of signal molecules into the rumen. The in vitro fermentation systems do not include a provision for removal of soluble substances except through the overflow. Accordingly, additional methods to investigate epithelial absorption processes are needed and are outlined in section 3.

2.3. Batch culture techniques

In vitro batch culture systems based on ruminal fluid have traditionally been used to estimate (total-tract) organic matter digestibility, and standardization and routine application of these methods have been discussed already more than 50 years ago (e.g. Alexander and McGowan, 1966). Of all in vitro batch culture systems, gas production techniques have attracted the attention of researchers to study not only ruminal feed digestibility, but direct or indirect impacts of animal production on the environment (Krishnamoorthy et al., 2005). This widespread attraction became notably evident in a comprehensive special issue published in Animal Feed Science and Technology in 2005, comprising 40 publications (Krishnamoorthy et al., 2005). Topics covered in reviews and research papers, as outlined in the preface to the special issue by Krishnamoorthy et al. (2005), comprised methodologies, repeatability, application to feed nutritive evaluation and feed secondary compounds, application to investigate environmental impacts, such as methane production, application to simple-stomached animals and humans, describing and predicting gas production and quantitative possibilities for gas data.

Application of a range of in vitro batch culture systems, including gas production techniques, to nutritive evaluation of food in the hindgut of humans and other simple-stomached animals was also reviewed at that time (Coles et al., 2005) and it was pointed out that, compared with techniques simulating ruminal events, in vitro digestion methods of relevance to human food evaluation lack standardization, in vivo validation and justification.

Generally, in vitro gas production techniques are a versatile tool to study ruminal digestion and microbial metabolism, notwithstanding that the potential is paralleled by limitations (Rymer et al., 2005). More recently, Yáñez-Ruiz et al. (2016) reviewed the applicability of in vitro batch culture experiments to assess enteric methane mitigation in ruminants and provided a range of technical recommendations to harmonize techniques for feed evaluation, assessment of rumen function and methane production. The wide applicability of in vitro gas production techniques to study ruminal events is also reflected in modifications of the method where ruminal fluid is replaced with buffered fecal suspensions from ruminants (Aiple et al., 1992) and other herbivores (e.g., horses; Can et al., 2009), which would allow replacing ruminally fistulated animals with intact animals.

Numerous batch culture studies have been conducted to screen the effects of bioactive substances (e.g. plant secondary metabolites) on the microbial activity (e.g. methane formation; reviewed by Lewis et al., 2013; Morgavi et al., 2010; Yáñez-Ruiz et al., 2016) and fermentation kinetics. However, similar to the continuous culture approach only few studies have been carried out to determine the effects of undesirable substances or contaminants, and those few studies focused mostly on fungal metabolites. For example, Asiegbu et al. (1995) and Mojtabehi et al. (2013) investigated the effects of aflatoxins and Jeong et al. (2010) the effects of deoxynivalenol on in vitro gas and SCFA production. Morgavi et al. (2013) explored the methane inhibiting effects of fungal secondary metabolites from Monascus spp. using a batch culture approach, and Akkaya and Bal (2012) determined the aflatoxin binding capacities of a Saccharomyces cerevisiae extract and mycotoxin adsorbents based on hydrated sodium calcium aluminosilicate. Using batch culture Hahn et al. (2015) tested 20 commercially available products for their detoxification ability towards deoxynivalenol and zearalenone.

Even fewer studies investigated whether the rumen microbial activity degrades or biotransforms the substance of interest. Caloni et al. (2000) demonstrated a low depletion rate of Fumonisin B1 of 12% and 18% after 72 h of incubation in batch culture. And Mobashar et al. (2012) quantified the microbial degradation of ochratoxin A by using the Hohenheim gas test. By applying antibiotics and fungicides the authors were even able to distinguish the degradation efficiency of individual microbial groups. Likewise, by using a certain centrifugation protocol Westlake et al. (1989) and Kiessling et al. (1984) demonstrated the degradation of several mycotoxins by bacterial and protozoal preparations from ovine ruminal fluid. However, although a quick degradation of toxic compounds can sometimes be quantified with batch culture, it does not always support the observed fermentation pattern. For example, although patulin was shown to be unstable in rumen contents, it was still highly toxic to the in vitro rumen fermentation (Morgavi et al., 2003). After 4 h of incubation the
concentration of patulin decreased by 50%, and it was hardly detected after 18 h. The same group could also demonstrate that gliotoxin, a mycotoxin often found in conserved forages, was unstable in the rumen environment with 90% disappearance at 6 h of incubation (Morgavi et al., 2004). Others, though, demonstrated the stability of some mycotoxins in the in vitro rumen. As such, recoveries of mycophenolic acid and roquefortine C, both mycotoxins of Penicillium section roqueforti, were 79% and 41%, respectively, after 48 h of incubation (Gallo et al., 2015). Likewise, aflatoxin B₁ and deoxynivalenol were not degraded by ruminal microbes in vitro (Kissling et al., 1984). Apparently, these compounds are quite resistant to microbial degradation or ruminal biotransformation and will likely reach the lower gut.

Only a number of studies investigated contaminants other than fungal metabolites in batch culture. For example, Majak and Cheng (1987) determined the rates of glycoside hydrolysis and hydrogen cyanide release of three different cyanogenic glycosides, and de Oliveira et al. (2010) investigated the ruminal degradation of the toxic protein ricin found in castor seed. Some countries have reported contamination of milk by heavy metals like lead (Pb). To test the Pb binding efficacy of herbal additives in the rumen, a batch culture approach was used by Nurdin and Susanty (2015). The authors showed that with Cuminum zedoaria, Cuminum manggo and Cuminum cyminum supplementation less dissolved Pb was found in the ruminal fluid after incubation, suggesting a possible transfer of bound Pb to the lower gut and excretion with the feces. Using in vitro batch culture Váradyová et al. (2006) showed that inocula of sheep that grazed on heavy metal contaminated pastures resulted in significantly lower fermentation output than a control inoculum. Also, by using ovine rumen fluid Craig et al. (1992) demonstrated the microbial degradation of pyrrolizidine alkaloids.

In summary, batch culture studies can be used to determine the degree of contaminant degradation or biotransformation and the contaminants’ effects on ruminal microbes and ruminal fermentation. Consequently, results from batch cultures studies can significantly help as a decision support for follow-up investigations. In comparison to the continuous batch culture approaches they are easier and faster to apply, resulting in larger numbers of replicates. Contrastingly, continuous culture studies might be advantageous to determine whether long term microbial adaptation towards certain substances can occur.

3 Models for the intestinal transfer of nutrients and contaminants

The epithelium lining the intestinal tract represents the first and major barrier for both, nutrients and contaminants. Regarding their anatomy and physiological function, the different segments of the gastrointestinal tract differ substantially within and between species.

For studying transepithelial transport processes, the Ussing chamber technique has to be evaluated as the only experimental approach which allows both, quantification of unidirectional flux rates of certain molecules across the epithelial barrier and the electrophysiological characterization of transport processes. It furthermore allows studying xenobiotic metabolism by intestinal epithelial cells through targeted or non-targeted analysis of xenobiotic metabolites. This technique has originally been introduced by Hans Ussing, a Danish Physiologist, for measuring ion transport processes across the frog skin (Ussing, 1949; Ussing and Zerahn, 1951). Since decades this technique has further been developed by including a computer-controlled voltage clamp unit, and it has regularly been used for studies on all gastrointestinal epithelial tissues in many species.

For start of each experiment intestinal segments are taken immediately after slaughter, carefully rinsed with physiological saline at 4°C and opened longitudinally. The Tunica mucosa is stripped of the muscular and serosal layer and the mucosal tissue is then mounted between the two halves of an Ussing chamber with an exposed surface up to 2 cm², thus forming a mucosal and a serosal compartment. Each side of the chamber is connected with a buffer reservoir, which is continuously gassed with carbogen (95% O₂, 5% CO₂). The buffer composition depends on each experimental condition; for mimicking physiological conditions commonly an isotonic buffer solution at pH of 7.4 is used on each side. In order to maintain the viability of the epithelial tissues throughout the incubation period additional glucose should be given at a concentration of 10 mmol/L to the serosal buffer solution used for incubating tissues from the small intestines. For hindgut tissues the mucosal buffer solution should contain acetate, propionate and butyrate at physiological molar proportions with an overall concentration of 60 mmol/L, ensuring an adequate supply of trophic compounds to the tissues.

The Ussing chamber is connected with a computer-controlled voltage clamp unit. Electrodes located close to the tissues measure continuously the transepithelial potential difference (PD). Under open circuit conditions definite currents are applied to the tissue at regular intervals which induce a short-term change in PD₁. Based on the Ohm’s law the transepithelial tissue resistance can thus be calculated.

Active transepithelial electrogenic transport processes generate an electric current which can be set to zero by introducing a respective short circuit current (Iₑ) by a further pair of electrodes. Under these conditions the Iₑ is a measure for all electrogenic transport processes.

When both, a chemical and an electrical gradient are eliminated the transport properties can be determined by measuring unidirectional flux rates from the mucosal to the serosal (Jₑm) and from the serosal to the mucosal (Jₑs) side of the tissue using radioactively labelled substrates. In the absence of any electrochemical gradient the unidirectional flux rates differ significantly and result in a significant net flux (Jₑ) when active processes are involved in either absorption or secretion.

Numerous studies have been carried out in recent years to identify the transport properties of intestinal phosphate (reviewed by Muscher-Banse and Breves, 2019) and gastrointestinal Ca transport in ruminants (Wilkins et al., 2012; Schröder et al., 2015). Furthermore, the effect of SCFA on electrophysiological and co-transport properties for Ca, Na or urea across the bovine and ovine rumen epithelium have been studied, respectively (Sehested et al., 1995; Uppal et al., 2003; Abdoun et al., 2010). The Ussing chamber technique has also been used to understand chloride secretion and intraepithelial metabolism of histamine after increased histamine exposure in both, the porcine colon as well as the bovine rumen.
(Aschenbach and Gäbel, 2000; Ahrens et al., 2003; Kröger et al., 2013 and 2015). Whereas the flux rates of electrolytes can appropriately be quantified based on radioactively labelled substrates, the exact measurement of organic nutrients or contaminants necessitates a more laborious approach, as most organic compounds can be subjected to intraepithelial metabolism. Thus, highly sensitive analytical methods are needed to quantify the mucosal uptake and serosal release, also of epithelially formed metabolites, and the potential tissue accumulation as a function of time. This approach has been successfully introduced for measuring transport processes of SCFA in the porcine hindgut (Herrmann et al., 2011). In addition, epithelial transfer of the colon carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was evaluated across the rat intestines (Nicken et al., 2013). Finally, the Ussing chamber technique has also been successfully applied to measure drug transfer in human intestinal tissues (Roehnthal et al., 2012; Sjöberg et al., 2013).

Alternatively measuring the specific radioactivity on the mucosal and serosal compartments can be applied, which can only be performed when preparative chromatography for the respective substrate is available.

The time period for incubation of different segments of the gastrointestinal tract is of high relevance for the data quality originating from the experiments. Whereas rumen epithelial tissues can be incubated for at least 6 – 7 h tissues from the small intestines can only be used for approximately 2.5 – 3.5 h. Viability time of tissues from the hindgut ranges between rumen and small intestinal tissues. In order to check whether sensible data have been generated, a tissue viability test needs to be performed after each experiment using secretagogues, such as forskolin, theophylline or carbachol. In addition, nutrients which are transported by electrogenic mechanisms can also be used to test the tissue viability.

Other in vitro incubation techniques such as the everted sac technique (Harmeyer et al., 1973) can still be used for absorption studies. With regard to mechanistic and regulatory aspects these techniques do not provide the advantages of the Ussing chamber technique, due to the fact that only the Ussing chamber technique includes the continuous control of tissue viability markers.

Besides the Ussing chamber technique, gut epithelial cell culture models might help to understand local metabolism of xenobiotics in gastrointestinal cells. Although epithelial cell cultures from ruminal epithelium have been successfully established (e.g. Stumpf et al., 2009; 2011; Kent-Dennis et al., 2020), they have very scarcely been used for analyzing xenobiotic metabolism or transport, yet. Most of the established ruminal epithelial cell culture models published are based on primary ruminal cells harvested from freshly killed animals. Thus, to further reduce the numbers of required animals, immortal cell lines are essential, as they can be a meaningful tool for testing e.g. drug metabolism and cytotoxicity (Allen et al., 2005). Immortal enteroctye cell lines have been established for several species including bovines. As such immortalized bovine epithelial cell lines from the rumen and small intestine have been established as a tool to study transport mechanisms of pathogens and to evaluate drug delivery via membranous epithelial cells (Miyaizawa et al., 2010; Zhan et al., 2019).

Another study established a sheep ruminal cell line to screen for possible interactions of transgenic feed and food compounds with the cells lining the rumen (Bondzio et al., 2008). Accordingly, ruminant cell lines can help to understand molecular mechanisms behind the metabolization of contaminants and possible toxifying or detoxifying processes at the gut lumen-host (systemic) interface.

4 **In vitro models for hepatic metabolism**

Once nutrients or contaminants have crossed the intestinal epithelium, either via active transport, passive transcellular diffusion or the paracellular route, they are further transported via blood to the liver, or, when first transported with the lymph, enter the systemic blood circulation through the thoracic duct. Therefore, these substances may be subjected to different biotransformation and excretion pathways or may accumulate in different organs including tissues used for human consumption. Understanding the fate of contaminants after the intestinal absorption is thus pivotal for the assessment of risks for both animal health and consumers’ safety.

The liver, generally considered as the most important metabolizing organ, consists of different cell types including hepatocytes, endothelial cells, stellate cells, Kupffer cells, pit cells, and bile duct cells. Under non-pathological conditions, hepatocytes account for approximately 80% of the liver mass (Gerlach et al., 1994). The transporter-mediated uptake of endogenous substances and xenobiotics from the portal blood plasma into the hepatocytes occurs mainly via their sinusoidal (basolateral) membrane. In the context of metabolism, this process is referred to as phase 0 (Döring and Petzinger, 2014). In humans, the transport proteins from the class of organic anion transporting polypeptides (OATP), organic anion transporters (OAT), carnitine transporters (OCT) and to a lesser extent the Na+/taurocholate cotransporting polypeptide (NTCP) are involved in the active uptake of compounds (Müller and Jansen 1997; Marin, 2012). In contrast to the state of knowledge in humans and experimental animals, little is known about the occurrence of uptake transporters in tissues of ruminants. It was shown that OATPs can also be found in tissues of cattle (Liu et al., 2013; Xiao et al., 2014). After uptake, hepatocytes are responsible for converting lipophilic (toxic) xenobiotics, such as diet and environmental pollutants (plant and fungal toxins, pesticides, herbicides etc.) and drugs, into water-soluble forms, so-called metabolites, which can be eliminated from the body via the excretory organs such as kidney and intestine. However, this process can sometimes activate previously non-toxic substances into toxic metabolites or increase the potency of already toxic substances. Accordingly, the liver not only has a high detoxification capacity, but an activating potential of xenobiotics has also been shown (e.g. pyrrolizidine alkaloids; Mattocks, 1986; Roeder, 2000; Wiedenfeld and Edgar, 2011; or aflatoxins; Kuilman et al., 2000; Alvarado et al., 2017).

Two phases of biotransformation of both endogenous and exogenous compounds have been identified. These phases are referred to as phase I and phase II reactions and are catalyzed by numerous phase I and phase II enzymes. Phase I reactions represent the first stage of the organism’s own detoxification system and are catalyzed by Phase I enzymes, such as cytochromes P-450 (CYPs), or other oxidoreductases. Often nucleophilic groups are introduced into the molecules, like e.g. hydroxyl groups. Further phase I reactions are hydrations and dehydrations. Phase II enzymes such as uridine diphosphate glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), sulfotransferases (SULTs) or amino acid transferases
conjugate nucleophile groups of xenobiotics or their oxidized phase I metabolites with highly ionized hydrophilic molecules such as glucuronic acid (He et al., 2010; Maul et al., 2012; Li et al., 2017a), glutathione (Larsson et al., 1994; Muluneh et al., 2018), sulfate (Smith and Shelver, 2002; Li et al., 2017a) or amino acids (Knights et al., 2007). For this purpose, phase II enzymes always require cofactors. The conjugation usually detoxifies the compound, however, in some cases the conjugates can also be toxic (Kaligutkar et al., 2005). Conjugates can be further metabolized or passed through an enterohepatic cycle. Otherwise, the excretion of phase I metabolites or conjugates can occur renally, biliary, by sweat or by breath.

Species differences in metabolism between humans and animal species are of great importance for the risk assessment of xenobiotics. If the metabolism of compounds in humans and animals varies qualitatively or quantitatively, this leads to different concentrations of the given compound and its metabolites in the target organ or in the food of animal origin. Consequently, sensitivity to the compound also differs between the target species at the same administered dose. For example, of the 57 human CYP isoforms known to date, CYP1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5, 3A7 are attributed relevance for the metabolism of foreign substances. The isoforms mentioned have a low catalytic specificity, are often inducible and show considerable intra- and inter-species differences in their enzyme activities (Guengerich, 1997). The known relative amounts relative levels (percent of total P-450) in the human liver are on average below 5% for CYP2A6, 2C19, 2D6, 10% for CYP2E1, 15% for CYP1A2, 20% for CYP2C8, 2C9, 2C18 and 30% for CYP3A4, 3A5, 3A7. CYP1A1 and CYP2B6 are expressed in smaller proportion or after induction. A question that arises frequently is the comparability of, for example, CYP from animal species with that of humans. For example, the levels of CYP in the liver of humans, rats and ruminants can vary considerably. The substrate selectivities and catalyzed reactions are influenced by minute differences in the amino acid sequence of enzymes. Identical isoenzymes of different animal species are called orthologous enzymes (Pelkonen et al., 1998; Dacasto et al., 2005).

An in vitro model for the metabolism of contaminants should reflect the in vivo situation of the liver as closely as possible. Besides using the perfused liver as described below, established in vitro models include liver slices (Viviani et al., 2017), primary hepatocytes (Ehrhardt and Schmicke, 2016; Witte et al., 2019), immortalized (transgenic) cell lines (Kuroda et al., 2015; Yoshioka et al., 2016) and subcellular fractions derived from liver homogenates. Genetically modified organisms can be used to produce cDNA-expressed metabolizing isoenzymes such as CYPs, UGTs or SULTs, which are referred to as supersomes (insect cell expressed recombinant enzymes) or bactosomes (E. coli expressed recombinant enzymes) (Asselfa et al., 1989; Kost et al., 2005).

Subcellular fractions, as from the liver, can be divided by differential centrifugation into the 9000 × g supernatant fraction (S9 fraction), cytosol and microsomes (von Jagow et al., 1965; Hubbard et al., 1985; Graham, 2002; Richardson et al., 2016). The S9 fraction contains both the enzymes present in the cytosol (e.g. SULTs, GSTs) and those bound in the microsomes (e.g. CYPs, UGTs). After a first cell lysis step a solution is obtained, which contains broken cells, small fragments of the plasma membrane and the endoplasmic reticulum, while leaving the organelles of the cell (e.g. mitochondria, nuclei, lysosomes) intact. This solution is also termed “lysat” or “homogenate”, which is then subjected to a series of centrifugation steps in an ultracentrifuge to be fractionated into several components.

The S9 fractions, which are also commercially available from a variety of species, can be further separated into microsomes and cytosol by another centrifugation step (1 h × 104'000 × g). The microsomal fraction consists of vesicles, that are formed from the endoplasmic reticulum, containing phase I enzymes such as CYPs, NADPH-cytochrome P450 oxidoreductases (CPRs), epoxide hydrolases, flavin-containing monoxygenases, carboxylesterases, amidases, as well as two classes of phase II enzymes namely UGTs and O-, N-, S-methyltransferases (Kedderis, 2018; Sanchez and Kauffman, 2010).

Microsomes are widely used under well-defined experimental designs to evaluate the metabolic stability and metabolite formation of a compound. Accordingly, they can be used to identify possible metabolization of undesirable substances, which enter the animal metabolism after being absorbed from the gut. For this, 0.05 to 100 µM of the substance of interest are incubated with a buffer containing the microsomes and cofactors at 37°C (Jia and Liu, 2007; Knights et al., 2016). The activity of the CYPs and UGTs, however, can vary depending on the buffer. In general, 50-100 mM Tris HCl (pH 7.5 at 37°C) or potassium or sodium phosphate buffer (pH 7.4) is well suitable, but UGTs appear more active in Tris buffers than in phosphate buffers (Boase and Miners, 2002; Engrtrakul et al., 2005; Argikar et al., 2016; Badée et al., 2019). Metabolism studies require the addition of cofactors for the target enzyme, e.g. 1-4 mM NADPH for CYPs or 2 mM uridine diphosphate glucuronic acid (UDPGA) for UGTs. The protein concentration of the enzyme solution is an experimental variable and can be adjusted depending on the application and susceptibility of the substrate to be metabolized (Jones and Houston, 2004).

Metabolization can be started by preheating the substrate, buffer and cofactors at 37°C and adding the cold enzyme fraction. An alternative proposal would be to preheat the enzymes, substrate and buffer and start metabolization with the addition of the cofactor. The reaction can be stopped at specified times by adding one to three times the volume of ice-cold organic solvent (e.g. methanol, acetonitrile optionally acidified with formic acid or ammonium formate). The samples should then be well mixed by vortex and stored for at least 20 min at −20°C to −80°C to facilitate precipitation of proteins and salts, which can finally be sedimented by centrifugation (Jia and Liu, 2007; Knights et al., 2016). The enzymatic degradation of the substrate and the identification of metabolites may be investigated using a variety of analytical techniques. Examples are liquid chromatography coupled to mass spectrometry, nuclear magnetic resonance spectroscopy, UV detection or radioactivity detection (Wen and Zhu, 2015; De Vijlder et al., 2018; Foroutan et al., 2019; Zhang et al., 2019; Thiel et al., 2019; Tolonen and Pelkonen, 2015).

Successful experiments have been conducted for numerous contaminants (e.g. Maul et al., 2012; Kolrep et al., 2018). The determination of metabolites of the mycotoxin aflatoxin B1 is a prominent example for the application of in vitro hepatic metabolism studies. Hayes et al. (1977) determined more than five phase I metabolites after incubation of aflatoxin B1 with bovine liver microsomes. After the metabolites had been identified in vitro, a feeding study with cows was conducted. Several tissues were examined for the presence of these metabolites. One of these metabolites, aflatoxin M1, was extracted with organic solvents from kidney, liver and mammary gland obtained from a cow receiving 7.31 mg/day aflatoxin B1 over a 14-day period.
The metabolism of various toxic pyrrolizidine alkaloids present in many plants belonging to the families of *Asteraceae* has also been studied using liver tissue fractions from cattle (Huan et al., 1998; Düringer et al., 2004; He et al., 2010; Fashe et al., 2015; Kolrep et al., 2018; Muluneh et al., 2018). *In vivo* studies with cows provide evidence that some of these pyrrolizidine alkaloids or their metabolites are transferred to milk (Dickinson et al., 1976; Johnson, 1976; Candrian et al., 1991; Hoogenboom et al., 2011). By knowing the *in vitro* liver metabolism and identifying metabolites it can be estimated, whether there is a need for analytical methods to detect these metabolites in food of animal origin (e.g. milk, meat). However, due to the often-lacking standards for the resulting metabolites, their discovery remains a challenging task.

In phase III of metabolism, the metabolites as well as unmetabolized substrates are transported by a transporter mediated efflux either via the basolateral membrane into the portal blood or via the canalicular (apical) membrane into the bile (Döring and Petzinger, 2014). These transport proteins belong to the superfamily of ATP-binding cassette (ABC) transporters. The multidrug resistance associated proteins (MRP) 3, 4 and 5 are expressed in the basolateral membrane of human hepatocytes. They transport their substrates back into the sinusoidal blood plasma. From there they also enter the kidney. The ABC-transporters expressed in the canalicular membrane of human hepatocytes are the Multidrug Resistance Protein (MDR) 1, MRP2 and the ABC sub-family G member (ABCG) 2 protein, also known as P-gp. Their substrates are released into the bile ducts, from where they pass through the bile into the duodenum and are excreted fecally (Müller and Jansen 1997; Marin, 2012). The data on the occurrence of efflux transport proteins in farm animals is as limited as on uptake transport proteins. Results exist about the expression of efflux transporter ABCG2/BCRP in tissues barriers of lactating dairy cows, sheep and goats (Lindner et al., 2013). The large data gaps regarding the transport mechanisms of xenobiotics in farm animal tissues were pointed out by several scientists (Martinez et al., 2018; Virlk et al., 2019; Rosa, 2020). At this point, we would also like to emphasize that there is an urgent need for research on this topic.

5 Ex vivo organ perfusion models

A challenge is clearly, that biotransformation processes are often a result of a complex interplay of different metabolic and cellular systems that cannot be comprehended with simple cell culture models. Especially, excretion pathways via bile, urine or milk, the putative re-absorption from the intestinal tract or further metabolism after initial hepatic biotransformation contribute to the complexity. Thus, extracorporeal, i.e. *ex vivo* perfusion models including whole organs or even body parts may help to generate required information about the fate of contaminants in the body without using animal experiments (Daniel et al., 2018). Such perfusion models have a long tradition in toxicological testing and to understand and improve the preservation conditions for organs intended for transplantation in humans. However, they may also be used to gain insight into the metabolism and transfer of contaminants. Additionally, these models often allow the organ collection from slaughterhouses, thus reducing the number of animals needed to be killed for experiments (Grosse-Siestrup et al., 2002).

5.1 Kidney and liver perfusion models

The isolated and perfused liver has been described for various animal species already almost a century ago (Plattner, 1924; Höber and Titajew, 1930; Haywood et al., 1945; Gunberg et al., 1955). The first model of a perfused bovine (calf) liver was described in the 1960s (Chapman et al., 1961). In contrast to the studies with the liver, studies with isolated perfused porcine and dog kidneys have been conducted even earlier to study factors influencing urine formation (Loebell, 1849; Starling et al., 1925; Nizet, 1975). Since, the set-ups, techniques and use of different perfusates have improved further. Today, studies are performed with porcine livers and kidneys from slaughtered animals due to their anatomical similarity to humans, their model role for circulatory death and their accessibility (Grosse-Siestrup et al., 2003; Dondossola et al., 2019). The isolated porcine or bovine liver or kidney can be perfused with modified artificial fluids or (heparinized) blood and allows to study the biotransformation of certain molecules of interest (e.g. contaminants), the excretion through the venous blood, urine or bile and possible adverse effects on hepatic or renal function. Sampling of arterial and venous fluids as well as bile at various time points allows for subsequent toxicokinetic modelling at whole-organ level.

5.2 The perfused bovine udder

A variable amount of systemically available contaminants penetrates into the milk. This process depends on the lipophilicity and the degree of ionization, because the milk pH with 6.5 to 6.7 is lower than the pH of blood. Because of this pH, partitioning of weak acids penetrating the blood milk barrier is very limited. Accordingly, the ratio between the ionized and the non-ionized form of the compounds is very high in the blood plasma. The penetration via the blood-milk barrier can be pre-estimated in *silico* considering the pH, pK-dependant partitioning phenomenon. In the past, Ziv and Rasmussen (1975), Shen-Tov et al. (1997) and others calculated the transfer rate of various compounds from blood plasma into the milk considering their pH, pK-dependent ionization in plasma and milk. The authors found a good correlation of the *in silico* calculated data with results of *in vivo* studies (concentration in milk samples of treated cows). It must also be considered, that inducible active transport processes take place additionally (Halwachs et al., 2013; Mahnke et al., 2016).

First perfusion models of the bovine udder have already been described in the 50s of the 20th century (Peeters and Massart, 1952; James et al., 1956; Verbeke et al., 1957). Since the isolated perfused bovine udder was described as a well-suited model to describe the distribution of antibiotics in the udder tissue (Kietzmann et al., 1993), various *ex vivo* studies have been performed during the last two decades. The tissue distribution of various locally administered β-lactam antibiotics, as well as the transfer of marbofloxacin and ceftiofurino into the udder tissue were studied (Ehinger and Kietzmann 1998, 2000a, 2000b, 2001, 2006; Kietzmann et al., 2008). Advantages of using the isolated perfused udder are that tissue samples as well as milk samples can be taken repeatedly at various time points without ethical limitation, allowing the quantification of a test substance being excreted via milk. A disadvantage is the relatively short duration of the tissue’s viability of up to about 8 h, which must be controlled during the time of perfusion. Suitable viability parameters, which are measurable in the
perfusion, are the glucose consumption, the lactate production, as well as the lactic dehydrogenase (LDH) concentration. Additionally, the cell viability can be determined also in tissue samples by cell viability tests. In most experiments by Ehinger and Kietzmann and Kietzmann et al. the isolated bovine udder was perfused with Tyrode's solution, which resembles lactated Ringer's solution, but contains magnesium, a sugar (usually glucose) as an energy source, and bicarbonate and phosphate instead of lactate. Generally, with perfusion with blood (heparinized and diluted blood) is possible. However, selecting the ‘ideal’ and most suitable perfusion medium should be based on the solubility of the test compound in the perfusion fluid.

Knowing the time-dependent blood plasma concentration of a test compound or its metabolites, the udder perfusion can be performed with its adapted concentrations to simulate realistic situations. Finally, knowing the tissue and milk concentrations allows calculating the test compound’s total amount, which is supposed to be eliminated via milk.

To perform a study using the isolated perfused bovine udder, medium sized udders of slaughtered healthy lactating cows are used. Directly after slaughtering, blood clots in the gland’s vessels are cleared using heparinized Tyrode’s solution.

In the laboratory, the udder can be fixed in a ‘natural’ position using a metal frame. The perfusion has to be started within minutes after insertion of silicone tubes into the large arteries of each udder half. The large veins are also cannulated to allow sampling and removal of the perfusate. Smaller veins are closed using artery forceps. After an equilibration period, the perfusion is continued with a fluid containing the test compound. Efflux samples (perfusion) as well as a milk equivalent can be collected repeatedly during the perfusion period. In addition to the concentration measured in the milk equivalent, comparing the concentration in the perfusion fluid with the concentration in the perfusate allows calculating the test compound’s amount which diffuses into the udder tissue.

Unlikely in vivo experiments on intact animals, the isolated perfused bovine udder model enables the investigator to retain control over various internal and external variables, such as type and compositions of the perfusate or a certain treatment before or during the experiment, e.g., the impairment or improvement of the organ physiology. Besides the limited duration of ex vivo studies on perfused isolated organs, isolated organs are deprived of nervous regulation and lymph drainage. Anyhow, several studies have used the perfused udder model successfully to investigate the transfer of various substances from the blood into the milk or the udder tissue (Ehinger and Kietzmann 1998, 2000a, 2000b, 2001, 2006; Kietzmann et al., 2008).

6 Integrating *in vitro* and *in silico* generated data using kinetic modelling

*In vitro*, *ex vivo* and *in silico* methods produce data about individual *in vivo* physiological processes describing the transfer of undesirable substances in ruminants. In order to integrate these data from individual methods into a coherent whole, a systems biology approach like kinetic modelling is required.

6.1 Toxicokinetic modelling

Kinetic modelling is a mathematical tool used to predict absorption, distribution, metabolism and excretion (ADME) of a substance of interest in live organisms. The substance may be a drug, in which case the term used is pharmacokinetics (PK), or a toxin or toxicant, in which case the term used is toxicokinetics (TK). Kinetic models describe the fate of a substance entering an organism by compartmentalizing the organism and using differential equations based on biochemical principles to describe the mass flows between various compartments and the chemical reactions happening within. These compartments may be ad-hoc groups of tissues and organs (Numata et al., 2014), or represent well-defined physiological structures, e.g., the extracellular space of the liver (Suvateeva et al., 2020). In the former case, the models are called compartment TK models and in the latter case they are called physiologically-based toxicokinetic models (PBTK). Techniques exist to reduce the complexity incurred on by including many physiological tissues and lump them together into simpler compartment models, bridging TK and PBTK models (Pilari and Huisinga, 2010). Both flows and reactions in the compartments are represented with corresponding parametric differential equations, which yield the change in amount or concentration in each compartment over time.

Interest in PBTK models is growing, as their ability to accurately reflect the physiology of the underlying modelled processes makes them more accurate (provided sufficient data is available). Modeling physiological processes explicitly may increase the predictive ability of PBTK models compared to empirical TK models that only consider such processes in a lumped and effective fashion. There is much potential in this field and research is progressing fast, especially in the field of animal health (Lin et al., 2016). PBPK/TK models have been successfully developed for ruminants (goats, cows) by Leavens et al. (2012) and Li et al. (2018). While the goat model by Leavens et al. (2012) was developed for veterinary medicinal purposes, the cow model was developed to ensure compliance with maximum residue levels of penicillin in milk.

In *in vitro*–to-*in vivo* extrapolation (IVIVE) in conjunction with PBPK/TK is an approach to bridge *in vitro* and *in vivo* data and to examine the key mechanistic determinations of the kinetics. PBTK modeling has also been used to address IVIVE for animal-free risk assessment (Fabian et al., 2019). In that study, an eight compartment PBTK rat model was developed and its predictive efficacy assessed by comparing it to relevant in vivo studies. The applicability of the approach was demonstrated, and its limitations identified. Kinetic models can be built using available knowledge about physical and computational chemistry, animal physiology (models of systems), animal experimental data (empirical models) or a combination thereof (hybrid models) (Bonate, 2011). To describe the fate of a substance in an organism with (PB)TK modeling, the optimal kinetic equation parameters must be estimated, i.e., the parameters that ensure the model equations follow a time trajectory consistent to what one would observe *in vivo*. Therefore, data from *in vivo* feeding experiments can be used and fitted to the model equations, thereby identifying the kinetic parameters of interest (Bonate, 2011).

As an alternative, one can use data derived from the literature, from *in vitro* models and from *in silico* prediction algorithms (computational toxicology) (Bolt and Hengstler, 2020) to obtain estimates for TK parameters, reducing or
eliminating altogether the need for animal experiments (Paini et al., 2019). This approach poses challenges but at the same time has much potential (Lin and Wong, 2017), both for the sake of animal and human health and for saving time and money in the risk assessment of new undesirable substances. Following, we present a summary of how (PB)TK models can be used as the final step to validate, complete and integrate the information gained from in vitro models of ruminants, such as the ones presented in the previous sections.

6.2 Integrating in vitro and ex vivo data using kinetic modelling

The PBTK models are powerful tools that can be used to simulate the change in concentration of a xenobiotic in tissues (local internal exposure) of interest upon (external) exposure. They can potentially be extrapolated across species, doses, routes of administration and compounds, as well as used for IVIVE (Li et al., 2017b; Fabian et al., 2019; Lin et al., 2020). An apt example of how PBTK can be employed to extrapolate the fate of xenobiotics from dairy cattle to other species can be found in MacLachlan (2009). Such models require detailed knowledge about chemical attributes (e.g., partition coefficients) and in the case of PBTK of physiological variables (e.g., cardiac output, organ weights, and blood flow rates) (Lin et al., 2020), some of which can be challenging to obtain without relying on in vivo data.

In the next paragraphs, the feasibility of determining such essential variables using in vitro, ex vivo and literature data will be discussed for each of the organs and tissues most relevant for assessing the kinetics of xenobiotics in ruminants exposed through the oral route (Fig. 1).

Rumen

The rumen compartment is of crucial importance when modelling the fate of xenobiotics after oral intake, not only because it determines the rate of digesta flow to the intestine, but also because of the large heterogeneity of gut microbiota, which may metabolize the modelled substance. The digesta flow can be estimated from physiological equations and animal science literature. Absorption into the blood can be estimated with data from Ussing chamber experiments. Predicting the chemical

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**Fig. 1: A representative example of a toxicokinetic model for ruminants**

The colored compartments represent the various organs or tissues; the flows of contaminants in and out of compartments are represented by straight lines and the chemical reactions happening in specific compartments by curved lines. These flows and reactions are governed by kinetic parameters, which are written next to the corresponding arrow. The concentration change of a contaminant (and potentially its metabolites) in each compartment is expressed in the form of a parameterized differential equation. One subscript letter: internal reactions (e.g., liver metabolization, microbiome-mediated reactions, etc.). Two subscript letters: mass flow from one compartment (first letter) to another (second letter). Three subscript letters: excretion mass flow from one compartment (first letter) followed by the ‘o’ for output (second letter), followed by the excretion route (last letter).

Legend: f, feed; x, exposure route; r, ruminal solids; j, ruminal fluid; i, intestine; b, blood; l, liver; m, muscle tissue; a, adipose tissue; k, kidney; u, udder; of, out feces; om, out milk; ou, out urine.
reactions that a substance may undergo requires a different approach. As discussed above, continuous or batch culture models can be used to investigate the metabolization of contaminants by the ruminal microbiota. If reaction rates for metabolites can be obtained, they can be included in the model, improving its performance and accuracy.

**Intestine**

Two key parameters for the intestine compartment are the absorption rate into blood and the rate of fecal excretion. The absorption rate from the intestine depends on a variety of factors, including the membrane’s permeability, the substance’s stability in the digesta and the time available for absorption (Cho et al., 2014). The stability can be assessed by using with incubation methods, as mentioned above. The time available for absorption is the transit time of digesta in the intestine, which again can be found in animal science literature. As with the rumen, the permeability of intestinal walls to a substance and epithelial metabolism can be investigated with the Ussing chamber technique. Fecal excretion rates can be calculated based on physiological equations and bioenergetics (Gabel et al., 2003). If the absorption rate of a substance into the blood and the frequency and amount of fecal excretion are known, it becomes possible to calculate estimate the substance concentration in both feces and blood.

**Adipose, muscle and blood-tissue compartments**

The blood-tissue compartment is frequently called the central compartment for good reason: it connects all other compartments in the (PB)TK model, reflecting the physical reality of the animal organism. The influx of substance from the GIT, which plays a significant role in determining the substance levels in blood, has already been described. Likewise, important are the flows to and from the remaining compartments. When storage processes take place, one has to consider peripheral compartments, relevant for substances that accumulate in kinetically slower tissues (such as lipophilic substances in adipose tissue or lead in bones). Muscle tissue may also be relevant for substances that can bind to proteins. An effective approach in such cases is the estimation of partition coefficients. A partition coefficient is the ratio of concentrations of a compound between two distinct phases (in this case tissues or groups of tissues) at equilibrium. To apply partition coefficients, it is unnecessary that the transfer kinetics for all processes are in equilibrium or steady state, but it is crucial that the local distribution kinetics between the two relevant phases is fast compared to other kinetic processes ( quasi-equilibrated state). The distribution of a substance from the blood to those tissues can be modelled as the diffusion between two phases of a solute, a process that may be adequately described by a partition coefficient. A range of such partition coefficient prediction algorithms exist, ranging from those that use a simple octanol-water partition coefficient to account for the transfer of lipophilic substances into the tissue (Hermens et al., 2013) to more complex formulations, where the biological tissue is represented by a complex mixture of biochemicals (Schmitt, 2008; Poulin and Theil, 2000). Several methods are available to predict partition coefficients, both in vivo and in silico (Graham et al., 2012). More sophisticated predictors of partition coefficients, like those based on the polyparameter linear free energy relationship (pLFER) (Endo et al., 2013), can be used and capture the complex thermodynamics of biological molecules. Such an approach has already been applied successfully by Savvateeva et al. (2020) for developing (PB)TK models of contaminants in growing pigs. Using partition coefficient prediction methods nevertheless has limitations, given that they fail to represent the differences in lipid types and other specific molecular interactions and non-covalent binding relevant for biological systems.

Specific binding to blood proteins can be studied using in vitro methods (MacManus-Spencer et al., 2010). For instance, serum albumin is the most important blood protein carrier for perfluoroalkyl acids (PFAAs) (Forsthuber et al., 2020). This binding can be explicitly built into PBTK models, where only the free, unbound fraction is available for exchange with other tissue compartments (Loccisano et al., 2013).

**Liver**

The liver has an essential role in the metabolization of chemicals in the body. It can act both as sink of xenobiotics (hepatic clearance of a chemical) or as a source (since some xenobiotics are biotransformed in the liver and only then become actively toxic). It is therefore important to establish whether and how a xenobiotic is processed in the liver and to determine hepatic clearance (the rate at which a substance is degraded or transformed in the liver). Theoretical models that describe hepatic clearance are available for humans (Laveé and Funk, 2007) and can be adapted to other mammals as ruminants. Information about the blood perfusion rate to the liver can be obtained from animal science literature (e.g. Lescoat et al., 1996). The actual liver reactions can be investigated either through ex vivo perfused liver models or in vitro hepatic models as described above. The liver biochemical reactions with their rate parameters can then be included in the PBTK model1, encompassing possible self-induced metabolism (Savvateeva et al., 2020). Like other tissues, the liver may contain proteins that specifically interact with and store xenobiotics.

**Kidneys**

The kidney compartment in the model predicts the fraction of substance excreted via urine. Although no in vitro model capable of simulating complete kidneys exists, there are other viable options to obtain data without relying on animal experiments, like the ex vivo perfused kidney model mentioned before, or pure in silico models. Kidneys are a challenging organ to model, since they are key not only to excretion processes but also to osmoregulation, which is subject to complex hormonal regulation and causes kidney activity to steadily change in response to internal and external stimuli. Nevertheless, it is possible to single out some physiological parameters of central relevance: glomerular filtration, tubular reabsorption and secretion. Depending on the modelled substance, reabsorption and secretion may be excluded from the model, whereas the glomerular filtration rate (GFR) is indispensable. In fact, GFR has been shown to be proportional to renal drug clearance in

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some human PBPK renal models (Jankü, 1993), making it one of the most relevant parameters for modelling renal excretion of xenobiotics. Although to date no computational model exists for ruminants, the advances in predicting human renal clearance and urinal excretion (Doddareddy et al., 2006; Huang and Isoherranen, 2018; Watanabe et al., 2019) suggest the feasibility of developing similar prediction models for ruminants.

Udder
The udder compartment can be modelled by knowing the blood perfusion rate and the milk production rate, available in animal science literature (e.g., NRC, 1988), as well as the other kinetic parameters quantifying the transfer through the blood-udder barrier (e.g., fraction of compound passing through the barrier). The latter can be experimentally investigated using the ex vivo perfused udder model by analyzing the perfusion fluid and the resulting milk equivalent for the substance.

6.3 Challenges and opportunities of using kinetic modelling to predict the transfer of contaminants into ruminant-derived food
There are still many challenges on the way to using kinetic modelling to integrate the outputs of the many in vitro, ex vivo and in silico methods discussed to accurately predict the transfer of undesirable substances in ruminants without the need for animal experiments. Although several studies could successfully generate predictive models for the transfer of xenobiotics in the animal body (Strikwold et al., 2017; Zhang et al., 2018; Cheng and Ng, 2017; Zhang et al., 2020), there is a range of issues to be addressed. The IVIVE represents a bottleneck in the development of models and the estimation of model uncertainties may be difficult without independent datasets for evaluation and calibration. In some cases, industry-generated in vivo data (for e.g., pesticides and veterinary pharmaceuticals) can be used as validation dataset to avoid further animal experimentation. The search continues for alternatives to the comparison of model output with data from in vivo experiments as a validation approach.

Another issue in the integration of in vitro generated data with in silico methods is the heterogeneity of the various methods’ data output: this increases the complexity of the task and poses problems like the propagation of systematic and random errors on measurements, which can be difficult to estimate.

Despite the current issues of relying solely on mathematical modelling to predict the transfer of contaminants in animals, there are encouraging signs, as research is advancing rapidly and the methods continue to improve. Not having to rely on animal experiments to predict the transfer of xenobiotics could speed up the risk assessment of new chemicals, while reducing the costs of experiments, contributing both to human and animal welfare.

7 Conclusions
Several of the here reviewed in vitro, ex vivo and in silico methods have been established decades ago and are applied in laboratories worldwide. However, the number of studies centered on the transport and metabolism of undesirable substances in the ruminant animal is still modest. Although the applicable methods can adequately simulate individual body compartments, the challenge of aligning all their outputs to follow a xenobiotic and its metabolites of interest along the animal body remains a challenging one. In silico approaches using toxicokinetic modelling offer the potential to integrate the data produced by a variety of methods and perform effective in vitro to in vivo extrapolation (IVIVE). The goal is to employ such methods to produce results comparable to in vivo experiments and to simulate diverse feeding and production scenarios. It represents a viable way to close the remaining gap between in vivo and in vitro results.

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Conflict of interest
The authors declare that they have no conflicts of interest.