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Quantitative evaluation of ruminal nutrient degradation using *in situ* and *in vitro* methods

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SUMMARY

Quantitative evaluation of ruminal nutrient degradation using *in situ* and *in vitro* methods

Studies on ruminally cannulated animals are expensive and time consuming. Furthermore they are limited to a few facilities and animal welfare issues make reduced numbers of animal experiments desirable. One alternative is to simulate rumen fermentation using long-term continuous systems, allowing investigation of a large number of treatments in a relatively short period of time. Lack of standardisation of continuous culture systems impairs the comparison of research data. Therefore, the focus of a comprehensive review as one major part of the thesis was to compile and compare technical and other methodical aspects of the semi-continuous culture system 'rumen simulation technique' (RUSITEC). Crucial or non-standardised characteristics were highlighted, standardisation of procedures was suggested and limits of standardisation were considered. Procedural steps which were evaluated encompassed choice and feeding of donor animals, type of feeds or rations, sampling time of inoculum and, further, the technical structure and procedure of the RUSITEC systems with a motor, pump, vessels and buffer solution. Moreover, the choice of incubation bags with regard to pore size and material, the incubation time, the experimental run period and the selection of experimental feed have an impact on the results. To improve the comparability of studies, specified technical standards will reduce the variability within and between different models of the RUSITEC system. Finally, standardisations were proposed to reduce variability in the design of simulation systems, variability in the rumen inoculum and to improve the comparability of RUSITEC research data.

Another major objective of the thesis was to directly compare the ruminal fermentation characteristics and degradability of two common forages, i.e., grass silage (GS) and maize silage (MS), by parallel application of a coupled *in vivo*-*in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system. Both forages were incubated in the RUSITEC system as well as fed to rumen-cannulated sheep in six independent runs of 20 days in total with 14 days of adaptation and 6 days of sampling. The degradability coefficients of dry matter, organic matter and acid detergent fibre were affected by the method (each $P < 0.05$), while neutral detergent fibre (aNDFom) degradability was not different between RUSITEC and *in situ* measurements ($P = 0.10$). Likewise, Pearson correlation coefficients confirmed the comparability of *in vitro* and *in situ* values for aNDFom degradability, being 0.54 ($P = 0.04$) and 0.78 ($P = 0.02$) for GS and MS, respectively. Regarding the fermentation profile, total volatile fatty acid (VFA) concentrations were much higher *in vitro* than *in vivo* ($P < 0.01$), likely due to the missing absorptive capacity of the RUSITEC system.

A comparison of absolute fermentation values between methods appears not feasible. However, the order of individual VFA proportions was similar between *in vivo* and *in vitro* and the correlations for both total and individual VFA further supported this congruency, especially for MS. The *in vitro* data appeared well comparable to the data from the coupled *in vivo*-*in situ* approach, especially for MS, with a high reproducibility in both methods.

Therefore, the RUSITEC system may represent a sufficient replacement for laborious *in vivo* and *in situ* measurements when assessing nutrient degradability and general fermentation characteristics of feedstuffs. Adjustments in *in situ* incubation times as well as the standardisation of the operation of the RUSITEC system may further increase the significance of this *in vitro* method in the future. Likewise, further research on diurnal fermentation patterns is encouraged to substantiate the present findings.

ZUSAMMENFASSUNG

Quantitative Bewertung des Nährstoffabbaus im Pansen mittels *in situ* und *in vitro* Methoden

Studien an pansenfistulierten Tieren sind mit einem hohen Kosten- und Zeitaufwand verbunden. Darüber hinaus sind sie nur an wenigen Versuchseinrichtungen möglich und aus Tierschutzgründen ist eine Reduzierung der Anzahl an Tierversuchen grundsätzlich anzustreben. Eine Alternative besteht darin, die Pansenfermentation mithilfe künstlicher Systeme zu simulieren, was u. a. die Untersuchung einer großen Anzahl von Behandlungen in relativ kurzer Zeit ermöglicht. Die mangelnde Standardisierung kontinuierlicher Kultursysteme beeinträchtigt bisher den Vergleich von Forschungsdaten. Daher lag ein Schwerpunkte dieser Arbeit darin, eine umfassende Übersicht zu technischen und methodischen Aspekten des semikontinuierlichen Kultursystems „Pansensimulationstechnik“ (RUSITEC) zu erstellen. Möglichkeiten und Grenzen der technischen und methodischen Vorgehensweise wurden validiert und Vorschläge zu einer Standardisierung erarbeitet. Verfahrensschritte wie die Auswahl und Fütterung der Spendertiere, die Art der Futtermittel bzw. Rationen, der Probenahmezeitpunkt des Inokulums sowie der technische Aufbau und das Handling der RUSITEC-Systeme mit Motor, Pumpe, Gefäßen und Pufferlösung wurden behandelt. Weiterhin hatten die Auswahl der Inkubationsbeutel hinsichtlich Porengröße und -material, die Inkubationszeit, die Versuchslaufzeit und die Auswahl des Versuchsfutters Einfluss auf die Versuchsergebnisse.

Um die Vergleichbarkeit von Studien zu verbessern, ermöglichen festgelegte technische Standards, die Variabilität innerhalb und zwischen verschiedenen RUSITEC-Systemen zu reduzieren. Abschließend wurde eine Standardisierung vorgeschlagen, um die Variabilität des RUSITEC-Systems zu vermindern und damit die Vergleichbarkeit der RUSITEC-Daten zu verbessern. Einen weiteren Schwerpunkt bildete der direkte Vergleich der Pansenfermentationseigenschaften und des Nährstoffabbaus durch parallele Anwendung eines gekoppelten *in-vivo*-*in-situ*-Ansatzes im Pansen fistulierter Schafe sowie des *in-vitro*-RUSITEC-Systems. Eingesetzt wurden zwei typische Grobfutter; Grassilage (GS) und Maissilage (MS). Beide Futtermittel wurden in sechs unabhängigen Läufen mit insgesamt 20 Tagen, davon 14 Tage Anpassung und 6 Tagen Probenahme untersucht. Die ruminale Abbaubarkeit der Trockenmasse, der organischen Masse und der Säure-Detergenzien-Faser (ADF) wurden durch die Methode beeinflusst ($P < 0,05$), während sich die Abbaubarkeit der Neutral-Detergenzien-Faser (aNDFom) zwischen RUSITEC- und *in-situ*-Messungen nicht unterschied ($P = 0,10$). Ebenso bestätigten die Pearson-Korrelationskoeffizienten die Vergleichbarkeit der *in-vitro*- und *in-situ*-Werte für die aNDFom-Abbaubarkeit; sie betrugen bei GS 0,54 ($P = 0,04$) und bei MS 0,78 ($P = 0,02$). Wahrscheinlich aufgrund der fehlenden Absorption im RUSITEC-System waren die Gesamtkonzentrationen flüchtiger Fettsäuren (VFA) *in vitro* viel höher als *in vivo* ($P < 0,01$).

Ein Vergleich absoluter Werte für VFA zwischen den Methoden scheint nicht möglich. Allerdings waren die VFA-Profile *in vivo* und *in vitro* ähnlich, und die Korrelationen sowohl für die Summe der VFA als auch für die einzelnen VFA untermauerten diese Kongruenz weiter, insbesondere bei MS. Die *in-vitro*-Daten waren insgesamt gut mit den Daten des gekoppelten *in-vivo*-*in-situ*-Ansatzes vergleichbar, insbesondere für die MS.

Das RUSITEC-System kann als ein ausreichender Ersatz für aufwändige *in-vivo*- und *in-situ*-Messungen bei der Ermittlung des Nährstoffabbaus und genereller Fermentationseigenschaften von Futtermitteln angesehen werden. Anpassungen der *in-situ*-Inkubationszeiten sowie die Standardisierung der Verfahrensweisen des RUSITEC-Systems könnten der Anwendung dieser *in-vitro*-Methode in Zukunft zugutekommen. Ebenso sind weitere Untersuchungen zu diurnalen Fermentationsmustern zu empfehlen, um die vorliegenden Ergebnisse zu festigen.

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ABBREVIATIONS

ADF	acid detergent fibre
CAESITEC	caecum simulation technique
cm	centimetre
COSITEC	colon simulation technique
CP	crude protein
DM	Dry matter
e.g.	Exempli gratia
g	gramm
GS	Grasssilage
h	hour
HGT	Hohenheim gas test
i.e.	Id est
L	litre
mL	millilitre
mm	millimetre
MS	Maizesilage
NDF	neutral detergent fibre
OM	organic matter
RUSITEC	rumen simulation technique
VFA	volatile fatty acids

CHAPTER 1

General introduction

The forestomachs of ruminants such as cattle, sheep, and goats are a very complex ecosystems with a diverse microbial community. In a symbiotic relationship, the microorganisms use the host animal's feed and produce volatile fatty acids and gases, and synthesize microbial biomass. This physiological process is the elementary component of the digestive process in ruminants. In agricultural research, it is possible to examine the physiological processes to address various research questions in ruminant species by using rumen-cannulated animals. In response to the increased public awareness of animal welfare and the aim of animal nutrition research to decrease the number of cannulated animals used for experimental purposes (GfE, 2017), further developments of research approaches according to the principles of Russell and Burch (1959) have been initiated. Furthermore, following the 3R principle - replace, reduce, refine - of Russell and Burch (1959), researchers are encouraged to develop alternative models to simulate *in vivo* processes as closely as possible.

Since farm animals are not comparable to other commonly studied animals such as mice (DFG, 2016) the use of farm animals in research is indispensable at present. However, studies with cannulated animals are expensive, time-consuming, and limited, and given the aspects related to animal welfare, it is desirable to reduce the number of such studies in the future. Worldwide, it is common to use *in vitro* systems to simulate the digestive and fermentative processes within the gastrointestinal tract of animals (Kajikawa et al., 2003; Rymer et al., 2005).

Apart from the lower experimental costs compared to animal testing, an additional advantage of *in vitro* simulation is the possibility to test higher, in some cases potentially toxic, levels of feed additives (Hristov et al. 2012; Klevenhusen et al. 2021). Furthermore, in contrast to *in vivo* experiments, these *in vitro* systems facilitate the investigation of a large number of treatments in a relatively short period (Czerkawski und Breckenridge 1977). Depending on the species, it is possible to use simulation systems for a variety of questions beyond those relating to ruminants, for instance in studying dogs (Kröger, 2009), pigs (Bender et al., 2001), or horses (Dill et al., 2007), if the focus is on the microbial colonization of the large intestine.

If laboratories want to use simulation systems, they have to keep donor animals to derive start inoculum for their systems (Klevenhusen et al., 2021). The host animals provide a relatively constant environment for the microorganisms, including a continuous intake of nutrients and water, regulated temperatures and mixing of the substrates, partially continuous bicarbonate-phosphate saliva buffer for buffering the volatile fatty acids produced during the microbial fermentation, and permanent removal of end-products or undigested particles.

To replicate this process in an *in vitro* setting, batch cultures and continuous culture systems were developed. The systems vary from simple bulk types with various volumes to sophisticated continuous-flow types with different stirrers, and in- and outflow systems. Systems were developed to achieve rumen-comparable conditions during relatively short-term incubations of a few days (Davey et al., 1960; Stewart et al., 1961; Bowie, 1962; Quinn, 1962) to a few weeks (Slyter et al., 1964).

Subsequently, such systems were further developed or modified with feeding the diets in bags (Weller and Pilgrim, 1974; Czerkawski and Breckenridge, 1977) or automatically (Hoover et al., 1976) achieves a slower rate of solid turnover, which, among other things, results in better attachment of protozoa to solid materials. Moreover, these systems allow to separate the solid and liquid turnover by using bags, varying the overflow, filtering the outflow, and using several turning wing and stirrer techniques for slow or layer-dependent intermixing.

Over the years, two systems have prevailed in this context: the dual-flow continuous culture system (Hoover et al., 1976) and the semi-continuous culture system (Rumen Simulation technique, RUSITEC) (Czerkawski and Breckenridge, 1977). Kajikawa et al. (2003) provided a general overview of artificial rumen systems after the 1980s and showed that RUSITEC (Czerkawski and Breckenridge, 1977) was mainly used in Europe whereas the second major system named after Hoover et al. (1976) was especially used in North America.

The design of artificial rumen systems such as RUSITEC offers a broad spectrum of possibilities to evaluate the effects of diet composition (Hildebrand et al., 2011), feed additives on the ruminal disappearance rate of feed, or address climate-relevant challenges in research concerning alternative nutrient sources such as macroalgae to reduce the methane production of ruminants (Díaz et al., 2017; Roque et al., 2019), microbial protein synthesis (Carro and Miller, 1999; Russi et al., 2002; Wischer et al., 2013), microbial structure (Soto et al., 2013), and ruminal fermentation (Carro et al., 2009). Additionally, the RUSITEC systems could be used as inoculum resources for a variety of *in vitro* systems in the future (Barbi et

al., 1993). For instance, the RUSITEC system could be used as a donor system for rumen inoculum and provide an adjustable inoculum tailored to specific research questions, thereby reducing the number of donor animals needed, and giving research institutes without animals the opportunity to use inoculum.

Substantial differences between these two approaches must be considered, when continuous culture system data are compared with *in vivo* studies. The passage rates of the fluid and solid phase, feed intake per rumen volume, and the accumulation of fermentation end products due to the absence of a rumen wall are diffferent (Stewart et al., 1961; Czerkawski, 1984), but the systems attempt to simulate this procedure with the outflow of inoculum. The RUSITEC system allows the use of additives and chemicals in amounts that would not be tolerated by animals and accepts the system's poor conditions without losing the stability of the fermentation (Czerkawski, 1984). However, despite all efforts to simulate *in vivo* conditions as closely as possible, a meta-analysis by Hristov et al. (2012) revealed that RUSITEC and continuous culture systems are characterized by lower total volatile fatty acid (VFA) and acetate concentrations, low counts or lack of ruminal protozoa, and lower organic matter (OM) and neutral detergent fibre (NDF) ruminal degradability. In addition, the variability of data obtained by using RUSITEC and continuous culture systems is much higher than for *in vivo* experiments (Hristov et al., 2012). Part of this variation is likely explained by the variability of the technical trial design of semi-continuous and continuous culture systems (Deitmers et al., 2022). Moreover, the question concerning the extent to which the results of *in vitro* and *in vivo* experiments are comparable remains unresolved.

However, comparative trials are often not conducted in a parallel design, where animals and *in vitro*-systems feed at the same time or receive the same starting material, i.e., inoculum (Martínez et al., 2010a; Martínez et al., 2010b). Comparability in feeding trials is dependent on using substrates and the resultant developed inoculum, which is influenced by technical aspects of acquiring inoculum for *in vitro* research (Belanche et al., 2019). The donor animals and the *in vivo* part of the comparative trials have their own dry matter intake, which cannot be fully regulated as is the case in *in vitro* systems.

In studies using a RUSITEC system, information about the technical design and procedure, including the handling of donor animals, is often lacking. This is especially noticeable concerning the diets fed to the donor animals (Lee et al., 2011). Moreover, although information on the time point of collecting rumen inoculum and its handling before starting the RUSITEC system is important (Li et al., 2009) this aspect is often lacking. A comparison

across several research institutes performed by Kajikawa et al. (2003) indicated substantial differences in RUSITEC technical design, e.g., vessel volume, dilution rate, and measure of the incubation bags. Currently, it is highly questionable if a fair and meaningful comparison of results from different studies can be made because of the complexity of the technical design of the RUSITEC (Czerkawski and Breckenridge, 1977), many of which may exert an effect on the measured variables and outcome of the study.

The key challenge of the RUSITEC system thus relates to standardisation to ensure a better comparability and consistency of the results. To standardise the RUSITEC method, it is necessary to realise comparative trials and to establish standard protocols for such experiments which should then be followed closely across laboratories.

While *in situ* methods have been reviewed and suggestions for standardisation have been made over time (e.g., Südekum, 2005; GfE, 2022), there is still a lack of research concerning the technical design of RUSITEC systems. Furthermore, examinations on the influences of these technical properties and comparison trials of in situ to in vivo approaches and between different national and international laboratories using RUSITEC are still lacking to date.

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CHAPTER 2

Scope of the thesis

Studies with animals are expensive, time consuming, bear restrictions to control conditions and are increasingly under public criticism owing to animal welfare issues. One opportunity to simulate rumen fermentation is to use complex in vitro models, e.g., continuous culture systems. The overall aim of this thesis was (1) to characterize the current procedural variations of the semi-continuous culture system `rumen simulation technique´ (RUSITEC); (2) to indicate critical and unsatisfactorily standardized characteristics of RUSITEC systems and convey guidelines for a standardization in the application of RUSITEC; (3) to directly compare the fermentation characteristics and degradability of two common forages, i.e., grass silage and maize silage, by parallel application of a coupled *in vivo-in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system.

An opportunity to simulate rumen fermentation are long-time semi-continuous or continuous culture systems, allowing the investigation of a large number of treatments in a relatively short period of time. In Europe, the semi-continuous culture system RUSITEC is the most common system. The advantages of these technique over *in vivo* approaches are, higher throughput of samples, flexibility to apply potentially toxic levels of feed additives, standardized feed supplementation and lower experimental costs. Other than simpler systems like batch cultures, the more complex *in vitro* systems offer the possibility to remove fermentation end products from the system and to maintain a stable microbial community for a longer period of time resulting in a closer simulation of the *in vivo* situation.

The first manuscript (Chapter 3) provides a systematic overview of studies using the RUSITEC with different technical applications. It generated opportunities and limitations of the method and draws attention to missing guidelines. Moreover, the continuing need for comparative research was determined and discussed. The second manuscript (Chapter 4) reports a parallel application of a coupled *in vivo-in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system to directly compare the fermentation characteristics and degradability of two common forages, i.e., grass silage and maize silage using six rumen-cannulated sheep and a six vessel RUSITEC apparatus.

Chapters 3 and 4 are manuscripts already published. They are formatted according to the layout and the instructions of the respective journal. However, the font has been adjusted to make appearance of this thesis consistent. Moreover, the numbering of tables in this document is continuous and does not correspond to the numbering of the respective manuscripts.

CHAPTER 3

Opportunities and limitations of a standardisation of the rumen simulation technique (RUSITEC) for analyses of ruminal nutrient degradation and fermentation and on microbial community characteristics

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A B S T R A C T

Studies on ruminally cannulated animals are expensive and time consuming. Furthermore they are limited to a few facilities and animal welfare issues make reduced numbers of animal experiments desirable. One alternative is to simulate rumen fermentation using long-term continuous systems, allowing investigation of a large number of treatments in a relatively short period of time. Lack of standardisation of continuous culture systems impairs the comparison of research data. Therefore, the focus of this review is to compile and compare technical and other methodical aspects of the semi-continuous culture system 'rumen simulation technique' (RUSITEC). Crucial or non-standardised characteristics are highlighted, standardisation of procedures is suggested and limits of standardisation are considered. The literature search yielded information from 93 studies published between 1977 and 2019 and comprised data from 111 individual technical variants and test protocols. Not only the development of semi-continuous culture systems, but also technical design and details and handling of the RUSITEC system were considered.

Procedural steps which were evaluated encompassed choice and feeding of donor animals, type of feeds or rations, sampling time of inoculum and, further, the technical structure and procedure of the RUSITEC systems with a motor, pump, vessels and buffer solution. Moreover, the choice of incubation bags with regard to pore size and material, the incubation time, the experimental run period and the selection of experimental feed have an impact on the results. To improve the comparability of studies, specified technical standards will reduce the variability within and between different models of the RUSITEC system. It does not appear advisable to restrict the species of the donor animal to cattle or sheep, yet the feeding of donor animals, the sampling time of inoculum, the preparation and the starter volume of rumen fluid should be specified in guidelines. Moreover, technical measures such as pore size of incubation bags, particle size of incubated feedstuffs and artificial saliva composition, as well as flow rate, need to be standardised. In contrast to standardised batch culture-based systems like the Hohenheim gas test, there are no uniform guidelines for the application of RUSITEC systems. Finally, we propose standardisations to reduce variability in the design of simulation systems, variability in the rumen inoculum and to improve the comparability of RUSITEC research data.

Keywords: Guidelines, *In vitro*, Methodical comparison, Rumen fermentation, Technical procedure

Abbreviations: ADF, acid detergent fibre; CAESITEC, caecum simulation technique; COSITEC, colon simulation technique; CP, crude protein; DM, dry matter; HGT, Hohenheim gas test; NDF, neutral detergent fibre; OM, organic matter; RUSITEC, rumen simulation technique; VFA, volatile fatty acids

1. Introduction

The rumen is a very complex ecosystem and it is difficult to study its function under strictly controlled conditions. Despite the problem of varying environmental effects, most of the research on rumen metabolism is conducted on cannulated animals. Studies with animals are expensive and time consuming. The variability and high costs have contributed to the development of *in vitro* methods for simulating ruminal fermentation. Moreover, increased public awareness of animal welfare and the aim of animal nutrition research to reduce the number of cannulated animals used for experimental purposes (GfE, 2017) have led to further development of *in vitro* methodologies according to the 3R-concept of Russell and Burch (1959). Compared with animal testing, a further advantage of *in vitro* techniques is the possibility to test higher, and in some cases potentially toxic, levels of feed additives (Hristov et al., 2012; Klevenhusen et al., 2021). In contrast to *in vivo* experiments, batch culture *in vitro* systems allow the investigation of a large number of treatments in a relatively short period of time. In addition to simple systems like batch cultures, complex continuous culture systems simulate removal of fermentation end products and maintain a stable fermentation for a longer period of time, e.g., up to 49 days (Czerkawski and Breckenridge, 1977), resulting in a closer simulation of the *in vivo* situation.

The design of artificial rumen systems offers a broad spectrum of possibilities to evaluate effects of diet composition (Hildebrand et al., 2011a), feed additives on ruminal disappearance rate of feed (Díaz et al., 2017), microbial protein synthesis (Carro and Miller, 1999; Russi et al., 2002; Wischer et al., 2013b), microbial structure (Soto et al., 2013) and ruminal fermentation (Carro et al., 2009). When comparing continuous culture system data with *in vivo* studies substantial differences between these two approaches must be considered. The passage rates of fluid and solid phase, feed intake per rumen volume and the accumulation of fermentation end products because of a missing rumen wall are not comparable (Stewart et al., 1961; Czerkawski, 1984). Batch cultures of ruminal microorganisms are simpler systems without constant inflow and no outflow. In contrast, the semi-continuous rumen simulation technique (RUSITEC) (Czerkawski and Breckenridge, 1977) and continuous rumen simulation systems (Hoover et al., 1976; Hannah et al., 1986) are characterised by variable in and outflows. Kajikawa et al. (2003) gave a general overview of artificial rumen systems from the 1980s and showed that the semi-continuous rumen simulation technique (Czerkawski and Breckenridge, 1977) was mainly used in Europe

whereas the second major system of continuous rumen simulation (Stewart et al., 1961; Hoover et al., 1976) was especially used in North America.

The RUSITEC system allows using additives and chemicals in dietary concentrations that would not be tolerated by animals and also accepts extreme rations creating poor ruminal conditions without losing the stability of the fermentation, e.g. 950 g straw/kg diet (Czerkawski, 1984). Despite all efforts to simulate *in vivo* conditions as closely as possible, a meta-analysis by Hristov et al. (2012) revealed that RUSITEC and continuous culture systems are characterised by lower total volatile fatty acid (VFA) and acetate concentrations, low counts or even lack of ruminal protozoa, and lower ruminal digestibility of organic matter (OM) and neutral detergent fibre (NDF). The variability among studies of data obtained by using RUSITEC and continuous culture systems was much higher than observed *in vivo* (Hristov et al., 2012). Part of this variation is likely explained by the variability or low degree of standardisation of the technical design of semi-continuous and continuous culture systems.

The aim of the present study is to critically review a frequently used system of long-term rumen simulation, namely the RUSITEC, and to evaluate and discuss the literature on methodical aspects. Articles published between 1977 and 2019 were searched for using the Google Scholar, ScienceResearch.com and Science Direct® platforms applying the following keywords alone or in various combinations: RUSITEC, rumen simulation, fermentation, rumen technique, *in vitro*, *in vivo*, semi-continuous simulation, rumen technical procedure, methodical comparison, *in vitro* vs. *in vivo* comparison, single flow continuous systems, dual flow continuous systems, rumen simulators, Czerkawski and Breckenridge, simulation of fermentation, ruminant simulation, continuous culture and continuous simulation.

This review describes details relating to the technical and methodical procedure of RUSITEC which reported at least two technical or methodical details. Furthermore, it indicates critical or non-standardised characteristics and identifies the opportunities and limits of a standardisation of this *in vitro* system.

2. Historical and technical overview

In addition to research on batch cultures, artificial rumen systems have been developed and advanced since the 1950s. The systems vary from simple bulk types with various volumes to complicated continuous-flow types with different stirrer, in- and outflow systems. Warner (1956) described the criteria for the construction of an artificial rumen and Adler et al. (1958) devised a constant-flow system where growth of rumen microorganisms could be measured.

Systems were developed to achieve rumen-comparable conditions during incubations ranging from a few days (Davey et al., 1960; Stewart et al., 1961; Quinn, 1962; Bowie, 1962) to a few weeks (Slyter et al., 1964). Because these systems had shown a time-dependent decrease of protozoa concentration, the focus turned to maintaining protozoal numbers in a long-term incubation trial in the 1970s. Subsequently several systems have been developed or modified respectively, where feeding the diets in bags (Weller and Pilgrim, 1974; Czerkawski and Breckenridge, 1977) or automatically (Hoover et al., 1976) achieved a slower rate of solid turnover, which resulted in better attachment of protozoa to solid materials.

Moreover, these systems separated the solid and liquid turnover by using bags containing the feed, by varying the overflow or by filtering the overflow. In the 1980s, Teather and Sauer (1988) and Fuchigami et al. (1989) used several turning wing and stirrer techniques for a slow or layer-dependent intermixing.

Over the years, two systems have prevailed: the dual-flow continuous culture system designed by Hoover et al. (1976) and the semi-continuous culture system RUSITEC developed by Czerkawski and Breckenridge (1977). The RUSITEC (Fig. 1) is originally based on a 1 L vessel containing buffered ruminal fluid, which is placed in a water bath, in general at a temperature of 39 °C to simulate the ruminants' rumen temperature. The vessels have an inlet at the bottom and a Perspex screw flat cover with two inlets. One inlet is for sampling at any time using a sampling valve and the other connects the fermenter with the overflow flask via tubing. The liquid overflow is collected within this flask, which is connected to a 5 L gas bag (Czerkawski and Breckenridge, 1977). To maintain anaerobic conditions, the whole system must be gas-tight. Polyethylene containers are placed inside the vessel, containing two nylon bags filled with feed. Originally, nylon bags with a pore size of 1000 µm were used by the inventors (Czerkawski and Breckenridge, 1977) and, one was filled with rumen solids the inoculation day and the other with treatment diets, respectively, at the beginning of the RUSITEC procedure. This approach allows for differentiation between solid and liquid contents within the vessels (Czerkawski and Breckenridge, 1977). After a 24 h interval it is a common work routine to open the vessels, change the rumen solid bag to a diet bag, then after a further 24 h the opposite bag is changed, resulting in an incubation time of 48 h per bag (Czerkawski and Breckenridge, 1977). Routinely, two nylon bags are placed within one vessel, but it is also possible to incubate three or four bags (Czerkawski and Breckenridge, 1977). This opportunity can be used to examine degradability at different times by leaving some nylon bags shorter or longer than 48 h inside the vessels (Czerkawski and Breckenridge,

1979b). The feed container is moved up and down by a stainless-steel rod through the flat cover of the vessels. A motor keeps it moving continuously with a speed of 20 rotations per minute and produces a vertical stroke of 50-80 mm at 8 cycles per minute, to generate a constant mixture with liquid fluid. Artificial saliva is infused constantly at the bottom of the vessel. This simple construction allows experimental runs from 4-8 weeks (Czerkawski and Breckenridge, 1977) and gives the operator maximum control (Czerkawski, 1984).

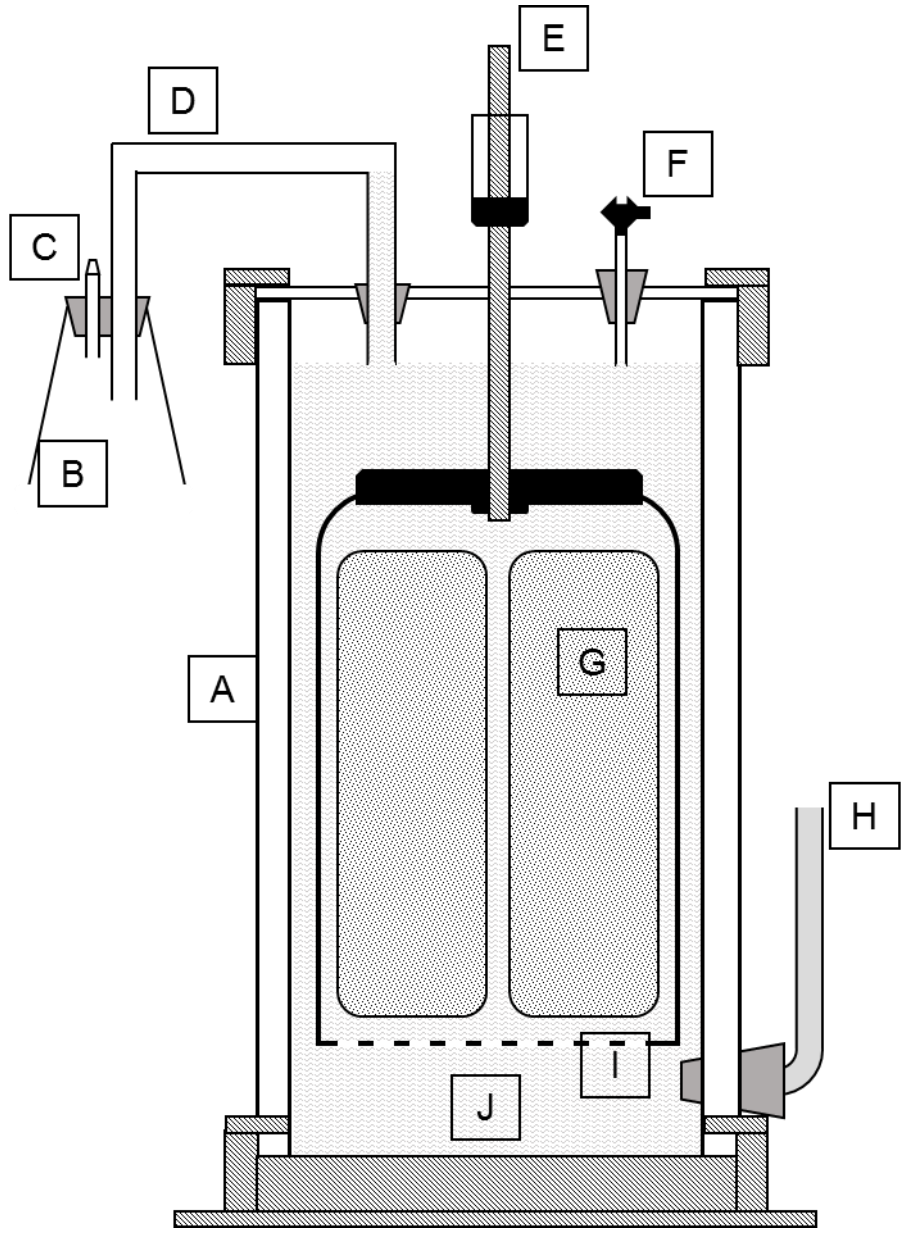

Figure 1 Schematic diagram of one unit of the RUSITEC system. A, main reaction vessel; B, vessel for collection of effluent; C, line to gas-collection bag; D, outlet through overflow; E, driving shaft; F, sampling valve; G, nylon gauze bag; H, inlet of artificial saliva; I, perforated food container; J, rumen fluid

3. Critical aspects of RUSITEC

3.1. General considerations regarding donor animals

Simulation of ruminal fermentation processes in continuous culture systems requires an inoculum to start the system. The activity and microbial composition of the inoculum for *in vitro* studies depends on donor animals, their diets, sampling time and handling of the inoculum (Mould et al., 2005). Table 1 summarises information in the literature on donor animals and their feeding and on handling of the inoculum. It is evident that the two main species of donor animals are sheep (56%) and cattle (42%), whereas goats and bison represent only 2%. Along with the more complicated handling of cattle, the barn capacity may be a limiting factor in research facilities, favouring the use of small ruminants as donors, such as sheep. However, Moon et al. (2010) showed an effect of animal species (goat, sheep and cattle) on rumen dry matter (DM) digestibility *in situ* and also Aerts et al. (1984) had shown the differences of cattle vs. sheep in digestibility of feedstuffs years before. On the other hand, Oss et al. (2016) reported synergism of cattle and bison inoculum on ruminal fermentation and select bacterial communities in a Rusitec study. Moumen et al. (2007), using a continuous culture system, compared goat and sheep inoculum and obtained higher values for carbohydrate digestibility and greater VFA production for the goat inoculum, whereas efficiency of bacterial protein synthesis was not affected by animal species. Also on aflatoxin B1 degradation, Upadhaya et al. (2009) indicated that goats demonstrated a higher aflatoxin B1 degradability compared with Holstein steers. Nevertheless, for the research and evaluation of feeds for dairy cows, often inoculum from sheep is used in the *in vitro* research.

Horton et al. (1980) observed that *in vitro* degradability of DM using straw-based inoculum (donor animals fed straw) was higher with sheep inoculum compared to cattle inoculum, whereas the inoculum from hay-fed cattle resulted in the highest degradability values. This was later confirmed by Holden (1999) who described that the diet of the donor animal may affect the degradation of OM and detergent fibre fractions more than the species itself. Also Boguhn et al. (2013) did not observe any effect of the donor animal on degradation of the fibre fraction and concluded that the diet of donor animals has a greater effect.

The data on *in vitro* fermentation and efficiency of microbial crude protein (CP) synthesis from Witzig et al. (2015) showed no significant differences when using either a hay-concentrate- or silage-based inoculum when incubating maize or grass silage in the RUSITEC. However, in a companion study using the same substrates in the RUSITEC and

the batch culture system Hohenheim gas test (HGT), Boguhn et al. (2013) found that there was an animal species effect on *in vitro* gas production and methane concentration in the HGT with higher values for sheep than cattle. Considering the results of degradation of proximate constituents and fibre fractions as well as amounts of VFA in liquid effluent, the authors concluded that the diet fed to donor animals affected fermentation and microbial CP synthesis more than the animal species itself.

Moon et al. (2010) showed an effect on *in vivo* rumen microbial populations and enzyme activities depending on animal species (goat, sheep and cattle). Whereas Li et al. (2009) indicated a greater influence of individual animals on *in vivo* bacterial diversity. Boguhn et al. (2013) used two animals per species in the RUSITEC which may reduce the specific effect of individual animals. However, this does not answer whether the RUSITEC can also be run with a single donor animal. Li et al. (2009) also detected that between-animal variation influenced the rumen microbial community more than sampling location or sampling time. Some authors observed *in vivo* different digestibility values between cattle and sheep (Südekum et al., 1995) and even between cattle and bison (Hawley et al., 1981a, 1981b), indicating that the microbial activity of rumen inoculum varied among species.

Notwithstanding the partly conflicting results of the above-mentioned studies, Klevenhusen et al. (2021), in a recent comprehensive review, evaluated studies which compared ruminal contents from different species in *in vitro* experiments and concluded from their evaluation 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 standardised and procedures are applied consistently regardless of the origin of ruminal fluid.

To reduce variation in the source of inoculum used for the batch culture system HGT, Rymer et al. (2005) recommended collecting inoculum from several donor animals. Most studies included in this review used three donor animals or more to inoculate RUSITEC fermenters (see Table 1). When there was more than one donor animal, inocula were pooled in 27 studies (e.g. Komisarczuk et al., 1987; Bogaert et al., 1989; Martínez et al., 2010a; Wischer et al., 2013a; Hartinger et al., 2019b). However, none of the studies documented pooling and filtering procedures in detail. Likely due to high costs for the management of cannulated donor animals and other constraints including extensive approval processes, twenty trials used only one donor animal for inoculum (e.g. Jalč et al., 1989; Carro et al., 1992, 1995; Hess et

al., 2003; Jaurena et al., 2005), which is not recommended mainly because the specific effect of individual animals would receive too much emphasis.

Table 1 Information concerning the donor animals and rumen inoculum in 93 published RUSITEC studies covering 111 different technical variants.

Item	N	Characteristic	Reference no. (see Appendix)
Animal number	84	Mean 3.5, SD 5.2, Median 3, Max 48, Min 1	1–4; 6; 5; 7–11; 14; 13; 15–19; 24–30; 33–48; 53–56; 58–61; 61; 62; 64–68; 71–81; 83–87; 89–93
Species	104	sheep (n=60), cattle (n=45), goat (n=2)	1–4; 6; 5; 7–12; 15; 16; 18; 23; 22; 20; 21; 19; 24–42; 44–48; 52–68; 70–78; 78; 80–84; 86; 87; 89–93
Breed	41	Meat, wool and crossbreed sheep (n=12), beef breed cattle (n=4), dairy breed cattle (n=24), dairy goats (n=1)	2; 3; 5; 7; 10; 11; 14; 18; 24–28; 33; 35–38; 42; 43; 45; 47; 52–55; 59; 62; 67; 68; 73; 75; 77; 79–81; 87; 91
Diet	94	hay-based (n=75), straw-based (n=5), silage-based (n=8), concentrate-based (n=7), pasture (n=2)	1; 2; 4; 6; 5; 7–12; 14; 13; 15–18; 23; 22; 21; 19; 24–30; 32–42; 42–48; 52; 53; 55; 57–60; 62–68; 70–84; 86; 87; 90; 92; 93
Feeding adaptation time	8	10-31 days adaptation	7; 11; 17; 33; 64; 66; 80
Feeding interval	27	two times (n=22), ad libitum (n=4), eight times (n=1)	2; 7; 10; 11; 14; 18; 19; 25; 35; 37; 38; 42; 43; 47; 55; 59; 60; 72; 73; 76; 80; 86; 91; 93
Rumen fluid removal information	34	before morning feeding (n=21), after morning feeding (n=10), hand-squeezed (n=1), vacuum pump (n=1)	6; 5; 8; 11; 14–16; 18; 25; 35; 37; 41; 43; 53; 58; 64–66; 68; 71; 74–76; 80; 81; 84; 86; 87; 91; 93
Handling with inoculum after removal	40	pooled inoculum (n=27), and filtered (n=22 e.g. cheesecloth)	6; 5; 7; 10; 11; 17; 19; 25; 26; 28; 37; 52–56; 58–60; 62; 64–68; 71; 74–76; 78; 80; 81; 84; 86; 87; 93; 93

3.2. Feeding of donor animals

In addition to the donor species, another source of variation is the composition of inoculum depending on the feeding of donor animals. Numerous authors used ruminal fluid taken from donor animals fed on restricted amounts of hay only, others from donor animals fed on hay with ad libitum intake and concentrate, and some authors used rumen fluid taken from donor animals fed on rations with a 50:50 hay to concentrate ratio (see Table 1). Further specifications of feeding donor animals were using ground barley (Zeleňák et al., 1994), lucerne hay (McMahon et al., 1999) and grass or maize silage (Carro et al., 1995; Lengowski et al., 2016). In addition to the variation in feeds or diets, often insufficient documentation on the ingredients and chemical composition of diets in studies makes it difficult to compare data on methods and results.

Martínez et al. (2010a) indicated a more closely simulated situation of *in vivo* fermentation in a RUSITEC system when the inoculum was from animals fed the same diets as the fermenters and when animals were fed high-forage diets compared with high-concentrate diets. Boguhn et al. (2013) showed that the diet of the donor animal affected the pH in the RUSITEC vessel, and was lower for a grass hay-based diet than for a silage-based (grass and maize silage) diet. The NH3-N concentration was lower in the vessels inoculated with rumen contents from animals fed silage (Boguhn et al., 2013). Nagadi et al. (2000) indicated differences in *in vitro* gas production parameters in a batch culture system based on the HGT when the diet fed to the donor animals was based on different concentrate to hay ratios (20:80, 40:60 and 80:20). The degradation of CP and NDF *in vitro* was not affected by the source of inoculum in a RUSITEC, whereas the acid detergent fibre (ADF) degradability was significantly lower when grass silage instead of hay plus concentrate was fed to the donor animals, likely mediated by an adjusted microbial community and activity (Boguhn et al., 2013). Furthermore, the *in vitro* VFA production was significantly affected by the diet of the donor animal (Boguhn et al., 2013). The results of Witzig et al. (2015) and Belanche et al. (2019) indicated that the inoculum is influenced by the diet consumed by the donor animals and that it affects the composition and activity of the microbial community *in vitro*. How strongly the microbial community is influenced by minimal differences in feeding is also shown in controlled *in vitro* experiments. Hartinger et al. (2019a) showed the influence of differently produced lucerne silages on the ruminal microbiota composition in the RUSITEC.

The effect of donor animal diet on the microbial community is reinforced by another effect: the specific adaptation of microorganisms to the conditions of the *in vitro* system. Carro et al.

(1995) observed a decreasing number of protozoa after starting incubation (RUSITEC) and Muetzel et al. (2009) found changes during the measurement period in the cellulolytic microbial community composition (continuous culture system) as well. In contrast, Strobel et al. (2008) concluded that the RUSITEC could sustain a microbial community with population sizes and phylotype diversities similar to those expected *in vivo*. However, Lengowski et al. (2016) detected the highest reduction in protozoal numbers within the first 48 h after the start of RUSITEC incubations. These results indicate that the vessel fluid in rumen simulation systems after the adaptation phase does not reflect the original population in the rumen of the donor animals. This is in line with RUSITEC studies of Martínez et al. (2010b) and Mateos et al. (2017) who analysed the evolution of bacteria, fungi and methanogenic archaea over the incubation period and observed that the microbial population changes significantly.

Summing up, before collecting rumen fluid and solids, donor animals must be fed a specific diet for a sufficiently long adaptation period to obtain an inoculum of stable activity. In general, there is little information about this process in RUSITEC publications (Table 1) but some authors describe adaptation phases of 14 or 31 days, respectively (Godoy and Meschy, 2001; Martínez et al., 2009; Martínez et al., 2010a; Witzig et al., 2015). To reduce variability between studies, collection of inoculum from more than a single donor animal, which must be adapted to the diets for at least 14 days, is firmly recommended. Feeding donor animals a hay-based diet, including two meals per day, such as in the HGT system is advised (Menke and Steingaß, 1988; VDLUFA, 2012).

3.3. Vessel volume, motor movement, inside pressure

The basis of an artificial technical system to simulate a rumen is the vessel or fermenter as experimental unit, in which all the fermentation processes are performed. Typically, the vessels are made of glass or plastic. The capacity of vessels also determines the amount of fresh rumen inoculum at the beginning, the amount of artificial saliva flow and various technical details such as the volume of effluent bottles, gas bags, motor power and heated water bath. The original RUSITEC system described by Czerkawski and Breckenridge (1977) had a fermentation vessel volume of 1 L. The variation in vessel volumes in the literature ranges from 0.5 L (Díaz et al., 2017) to 1.1 L (Makkar and Becker, 1995; Wettstein et al., 2000) and even 1.4 L (Czerkawski and Breckenridge, 1979a; Table 2). Generally, the volume of RUSITEC systems has not changed much in the past 20 years (Kajikawa et al., 2003) and is more uniform than other artificial continuous rumen systems, which generally range between 0.5 L (Fuchigami et al., 1989) and 4 L (Hoover et al., 1976). Exceptions are systems

with volumes of 18 L (Aafjes and Nijhof, 1967) and in a whole rumen content macro system (40 L; Udén, 2011).

A motor is required for the vertical movement of the feed containers and should be selected depending on the number of vessels and their volume. Czerkawski and Breckenridge (1977) described that the motor produced a vertical stroke of the feed containers of 5 to 8 cm at eight rotations per minute. In the literature, the information in RUSITEC trials about the motor movement is limited (Table 2). This approach prevents the formation of different layers inside the vessel, as in other artificial rumen systems (Fuchigami et al., 1989). The solid retention time can only be controlled by different incubation times of the feed bags (Martínez et al., 2009). A vertical stroke of 5 to 7 cm (Bogaert et al., 1989; Klevenhusen et al., 2015) and 7 to 8 rotations per minute (Stanier and Davies, 1981) were reported. Studies on varying the speed of the vertical movements in RUSITEC systems are not known, but a faster movement should be avoided to prevent mechanical washout losses of feed particles. The pressure inside the fermentation vessels is determined by feed container movement, gas production and mechanical drag of the effluent tubes. When the inflow of buffer solution occurs through long tubes at the bottom of the vessel, the inside pressure should be taken into account. Depending on the technical design, this may affect the effective buffer supply and could result in over- or under-buffering of the vessel contents.

To make comparisons of the results from different RUSITEC systems possible, it is important to take into account the buffer supply and the volume of starter medium in relation to the vessel volume. To generate an adequate and comparable volume for fermentation, gas production and sufficient space for handling incubation bags, it is advisable to choose an effective fermenter volume with a minimum of 800 ml.

Table 2 Information on technical variables concerning the RUSITEC vessels in 93 published RUSITEC studies covering 111 different technical variants.

Information	N	Mean	SD	Median	Min	Max	Reference no. (see Appendix)
Number of vessels	106	6.3	3.5	6	2	16	1–4; 6; 5; 7–12; 14; 13; 16; 17; 23; 22; 20; 21; 19; 24–41; 43–46; 48–80; 83–86; 88; 87; 89–93
Volume of vessel, L	96	0.87	0.1	0.85	0.5	1.4	1; 2; 4; 6–11; 14; 13; 16; 17; 23; 20; 21; 19; 24–30; 32–41; 43–48; 52–67; 70–74; 76–86; 88; 87; 89; 90; 92; 93
Motor movement	9	5-8 cm vertical stroke and 7-8 cycles/min					7; 8; 19; 54; 78

3.4. Pore size of incubation bags, bag material and washout losses

Table 3 gives an overview of technical details of the used incubation bags. The first RUSITEC trials were run with bags having a pore size of 1000 µm (Czerkawski and Breckenridge, 1977), whereas now pore sizes of 50, 100, 150, 200 and 1000 µm are commonly used. Most researchers have used nylon bags and, other than in rumen *in situ* studies, polyester was rarely used (Lee et al., 2011; Oss et al., 2016), although both materials are characterised by lightness, dimensional stability and tear resistance. Carro et al. (1995) studied the effects of different pore sizes of 40, 100 and 200 µm with a ration based on freeze-dried and chopped grass and maize silages. Protozoal numbers ($P<0.05$) and the disappearance (%) of DM and NDF ($P<0.01$) were higher with the 100 µm bags compared to 40 µm bags. There was no effect on the DM losses from the bags nor on the DM in the effluent and vessel fluid, when bags of both pore sizes were incubated only in artificial saliva (Carro et al., 1995). The observed differences between pore sizes of 40 and 100 µm, could be an indication of washout loss and thus used for a correction, similar to the one applied in in situ trials (Südekum, 2005). In a second trial from Carro et al. (1995), a comparison of 100 µm to 200 µm bag pore size showed that DM and NDF degradability were higher with the 100 µm bags. Methane and acetate production were also higher in the 100 µm pore size bags. Carro et al. (1995) detected large protozoa in bags with pore sizes of 100 µm and 200 µm,

respectively, but none in the 40 µm bags, which indicated that the pore size influenced the presence of protozoa. Also Meyer and Mackie (1986) indicated that the pore size may influence the population of bacteria and protozoa, especially large protozoa. For example Isotricha intestinalis, Isotricha prostoma and Eudiplodinium maggii were unable to enter bags with a pore size smaller than 53 µm. In a meta-analysis, Hristov et al. (2012) detected that, compared to the rumen, continuous culture systems generally have extremely low counts or a complete lack of ruminal protozoa, whereas non-RUSITEC simulation techniques typically show higher protozoal counts. This may be a consequence of solid feed supplementation without the use of incubation bags. Furthermore, it should be considered that Carro et al. (1995) chopped the diet to a length of 0.5 cm after freeze-drying, whereas other users have ground the complete diets through a 1-mm screen aperture (Boguhn et al., 2013; Wischer et al., 2013a; Lengowski et al., 2016). With a bag pore size of 1000 µm, hay or silage cut to a length of 2 to 5 cm were used in some studies (Jasper, 2000; Chawanit, 2003; Hartinger et al., 2019b). Chewing activity of animals was simulated with different particle sizes for concentrates (ground through a 3 mm sieve aperture) and forage (0.5 cm particle size) (Martínez et al., 2010a).

In conclusion, the results indicate that the pore size of the bags used for RUSITEC incubations influences the microbial population and therefore the degradability of the diet and fermentation of nutrients. For a sufficient evaluation, the pore sizes should always be reported. Recommendations concerning particle size should differentiate between coarse forages and concentrates. Single feeds or thoroughly mixed ration components, respectively, should be incubated in nylon bags preferentially with a pore size of 100 µm.

Table 3 Information on the technical variables concerning the RUSITEC incubation bags in 93 published RUSITEC studies covering 111 different technical variants.

Item	N	Mean	SD	Median	Min	Max	Reference no. (see Appendix)
Number of incubation bags	85	2.2	0.6	2	1	4	1–4; 6; 5; 7–12; 14; 13; 15; 16; 18; 20; 21; 21; 19; 25–30; 32; 33; 35–41; 43; 44; 47; 48; 52–56; 58–60; 62; 64; 66; 68–77; 79; 81; 83–86; 88; 87; 89–93
Bag material	96	Nylon					1–4; 6; 5; 7–11; 14; 13; 15–18; 20; 21; 19; 24–41; 43–57; 59–62; 64–73; 76–79; 81–86; 88; 87; 89–93
Bag material	2	Polyester					58; 74
Pore size of incubation bag, µm	91	228	307	100	40	1000	40 µm: 6; 14; 48; 58 48 µm: 30 50 µm: 7; 8; 60; 67; 72–74; 76; 81, 53 and 56 µm: 28; 84; 69 100 µm: 2; 5; 9–11; 14; 13; 15; 17; 25–27; 32; 34; 38–41; 43; 56; 59; 61; 62; 64–66; 85; 86; 88; 87; 89; 90 150 µm: 4; 24; 29; 36; 52; 54; 55; 71; 81 200 µm: 1; 14; 44–47; 49–51; 53; 91; 92 1000 µm: 18; 20; 21; 19; 35; 37; 47; 70; 91
Incubation bag content, g dry matter	80	13.1	3.9	14.4	3	21	1–4; 6; 5; 7–12; 14; 13; 16–18; 20; 21; 19; 28–35; 37–41; 43–49; 51; 53; 56; 58–60; 62–64; 66; 67; 69–79; 81–86; 88; 87; 89–93

3.5. Starting the RUSITEC

Before starting the RUSITEC, rumen fluids and solids have to be collected from the donor animals. Collection of rumen inoculum was conducted 2 h (McMahon et al., 1999; Neumann et al., 1999; Russi et al., 2002), 2.5 h (Gresner et al., 2015), 3 h (Wulff, 2001; Krause, 2002; Chawanit, 2003) or 6 h (Bogaert et al., 1990) after feeding.

Contrary to these observations, Menke and Steingaß (1988) indicated that sampling rumen contents just before feeding reduced the variation in composition of the inoculum in the HGT system. Li et al. (2009) indicated a similar structure of the bacterial community, when using different locations in the rumen and sampling times. In addition, the authors showed little impact on pH and total VFA in the rumen between sampling times. Collecting rumen inoculum before the morning feeding can also be seen in most RUSITEC publications (Table 1). In addition to the sampling time, the collecting procedure may have an effect on the quality of the rumen liquid and solids, but this process is rarely described in RUSITEC studies. Rumen fluid may be collected via a tube by suction followed by using hydrostatic pressure as with communicating vessels. Otherwise an electrical vacuum pump may be used or rumen solids are squeezed out (Table 1). However, the method used to obtain rumen fluid may influence the bacterial community in quantity and activity, respectively, as Rymer et al. (2005) have indicated for *in vitro* gas production techniques.

Information about the influence of rumen solids in RUSITEC systems in response to, e.g., donor animal feeding has not been reported. The treatment of rumen fluid after collection is also critical. During preparation and handling, oxygen and differences in temperature may affect the biological and physiochemical state of the rumen fluid. Nevertheless, most studies are limited to the information that the rumen fluid was filtered through two to four layers of cheesecloth (Table 1). To what extent the collection and preparation process affect the composition of the inoculum and comparisons among studies is largely unknown. Dehority (1984) highlighted that the number of protozoa was influenced by straining or squeezing through one or two layers of cheesecloth. In addition, the generic distribution of protozoa in rumen fluid obtained by squeezing or straining differs from generic distribution in whole rumen contents (Dehority, 1984).

The first days of a RUSITEC run are considered an adaptation phase. At the start, Czerkawski and Breckenridge (1977) reported a ratio of 0.625 rumen fluid, 0.25 buffer and 0.125 distilled water, respectively. This ratio was not applied in all studies reviewed and may need to be

standardised or adjusted in future studies (Table 4). Lengowski et al. (2016) recommended that the adaptation phase should last for at least 48 h to achieve a steady state of the microbial population. Kajikawa et al. (2003) reported an average of 7.9 days and a median of 7.0 days of adaptation time for RUSITEC trials. The present data set (Table 4) reveals a range in the adaptation phase from 3 days (Czerkawski and Breckenridge, 1979b; Machmüller et al., 1998; Wettstein et al., 2000; Hess et al., 2003; Engelmann et al., 2007; Vosmer et al., 2012) to 17 days (Jayasuriya et al., 1988; Newbold et al., 1996; Russi et al., 2002; Tejido et al., 2002), resulting in a mean and median close to values reported by Kajikawa et al. (2003). To further reduce the number of animal trials required, it is advisable to investigate the use of vessel fluid or effluent from an established RUSITEC system for inoculation of other *in vitro* systems. Barbi et al. (1993) used a RUSITEC vessel or effluent fluid to replace rumen fluid in *in vitro* forage-digestion techniques and confirmed the potential of this method for lessening the need for cannulated animals. The potential to substitute rumen fluid from donor animals with rumen fluid from a RUSITEC used for *in vitro* degradability of forage samples was indicated by Tejido et al. (2002). Recently, Spanghero et al. (2019) have compared rumen inoculum collected from cows at slaughter or from a continuous fermenter and concluded that continuous fermenters might generate inoculum for *in vitro* purposes and that short-term refrigeration was a valuable storage system to facilitate inoculum transfer between laboratories. These findings might attenuate the need for frequent collections from animals and would thus help to further reduce the number of experimental animals.

Technical and methodological aspects of starting RUSITEC systems should be harmonised to ensure comparability of results. Assuming that sampling time of rumen contents is of special importance, we suggest collecting rumen fluid before the morning feeding to reduce variability in its composition. An electrical vacuum pump with defined vacuum and only one layer of cheesecloth to separate bigger particles should be used. Under a uniform procedure, transport containers should maintain a stable temperature of the inoculum and avoid contact with oxygen. Thermos flasks filled with warm water (body temperature) have proven to be useful, if a quick transfer of inoculum via vacuum pump is possible. Further, it should be examined whether vacuum bottles are suitable for the transport of inoculum, because using thermos flasks includes the risk of gas creating excessive pressure.

Table 4 Information on initialising the RUSITEC in 93 published RUSITEC studies covering 111 different technical variants.

Item	N	Mean	SD	Median	Min	Max	Reference no. (see Appendix)
Incubation time, h per bag	81	48	5	48	24	72	1–4; 6–12; 14; 13; 15; 17; 18; 20; 21; 19; 24–33; 35–41; 45–48; 52–55; 58–62; 64; 66–68; 70–77; 79; 81–86; 88; 87; 89–93
Changeover phase time, h	81	25	6	24	24	72	1–4; 6; 5; 7–12; 14; 13; 15–18; 20; 19; 24–30; 32–41; 43; 45–48; 52–55; 58–60; 62; 64; 66–68; 71–77; 79; 81; 82; 84–86; 88; 87; 89–93
Changeover phase washing procedure, mL	21	63	25	50	40	140	3; 10–12; 17; 20; 19; 32; 35; 37; 40; 41; 47; 57; 60; 86; 91
Period per run, days	96	18	8	15	8	54	1–4; 6; 5; 7–13; 15–18; 23; 22; 20; 21; 19; 24–27; 29–45; 47–49; 51–68; 70–74; 76; 78–81; 83–86; 88; 87; 89–93
Adaptation phase, days	67	7	3	7	3	17	1; 2; 4; 6; 5; 7–13; 15; 18; 23; 22; 20; 21; 24–30; 32–38; 40; 43–45; 47; 49–53; 55; 61–63; 68; 71; 73; 74; 76; 78–82; 85; 86; 88; 87; 91–93
Main phase, days	65	9	5	7	4	25	1–4; 6; 5; 7–10; 12; 13; 15; 18; 23; 22; 20; 21; 24–27; 29; 30; 32–38; 40; 43–45; 47; 49; 51–53; 55; 60–63; 67; 68; 71; 73; 74; 76; 78; 79; 81; 82; 85; 86; 88; 87; 91–93
Starter medium							
ratio rumen fluid/ vessel volume	58	0.6	0.2	0.5	0.2	1.0	1; 2; 4; 6–8; 10; 14; 15; 17; 22; 20; 21; 19; 24–28; 30; 33; 35–38; 47; 48; 52–62; 64–66; 74; 77; 78; 81–86; 88; 87; 93

ratio buffer/vessel volume	47	0.3	0.1	0.2	0.1	0.6	2; 6; 8; 10; 14; 15; 17; 20; 21; 19; 24–27; 33; 35–38; 47; 48; 52–62; 64–66; 74; 77; 85; 86; 88; 87; 93
ratio dest. water/ vessel volume	15	0.1	0.0	0.1	0.1	0.1	8; 14; 15; 20; 21; 19; 33; 35; 37; 47
ratio rumen content/ vessel volume	57	0.08	0.03	0.01	0.01	0.16	1; 4; 6–11; 14; 15; 17; 20; 21; 19; 25; 28; 30; 33; 35; 37; 38; 40; 43; 45–48; 53; 55; 57–60; 62; 64–66; 71; 77; 78; 81–85; 88; 87; 89; 90; 93

3.6. Buffer pump, artificial saliva flow and dilution rate

A constant flow rate of artificial saliva (Table 5) is generally maintained by different types of peristaltic pumps (Czerkawski and Breckenridge, 1977; Jaurena et al., 2005; Hartinger et al., 2019b), which are perfectly suitable for continuous operation, require no valves or pistons and are useful for conveying fluids. Problems can arise from intensive tube wear, abrasion of tube material and a non-constant delivery rate. Moreover, location of the inflow into the vessel on the top or at the bottom may influence the temperature of the buffer entering the fermenter affected by the warm water bath. Additional disturbance arising from the precipitation of artificial saliva has often been found in our laboratory, such that intensive monitoring seems necessary, otherwise a variable buffer composition reaches the vessels. Besides the inflow of artificial saliva, its composition and flow rate significantly affect degradation and fermentation processes in the RUSITEC. Most often the artificial saliva composition developed by McDougall (1948) is used or forms the basis for buffer composition (Table 5). It is based on NaHCO3 (9.80 g/L), Na2HPO4 • 12 H2O (9.30 g/L), NaCl (0.47 g/L), KCl (0.57 g/L), anhydrous CaCl2 (0.04 g/L) and anhydrous MgCl2 (0.06 g/L) (McDougall, 1948). Lee et al. (2011) used NaHCO3 and Na2HPO4 at slightly different concentrations, Dong et al. (1997) omitted CaCl2 and MgCl2 and McMahon et al. (1999) and Oss et al. (2016) supplemented ammonium sulphate. Several authors used the isotope 15N as a marker tool for determination of microbial CP synthesis which should not, however, impact the buffering characteristics (Table 5). If experimental treatments used high proportions of maize silage or similar products with low CP content, the addition of urea reduced the deficit in available N (Hildebrand et al., 2011a; Hildebrand et al., 2011c). Tejido et al. (2002) and Carro et al.

(2009) used a modified McDougall artificial saliva related to NaHCO3 and Na2HPO4 concentrations to achieve a pH value similar to that found before feeding in the rumen of donor sheep fed a high forage (80:20 forage to concentrate) or low forage (20:80 ratio) diet. This approach appears plausible to achieve a better rumen simulation than by using the common, standard buffer but it weakens the comparison across studies, because the variability of diets is high and would also lead to a high variability in buffer compositions.

To our understanding, the buffer described by McDougall (1948) should be used as standard. Exceptions should only be made for fluid or water-soluble supplements as well as supplementation of deficient feed-additives and soluble markers. The quantity of added buffer can be expressed as buffer flow rate per day (relation of buffer flow to vessel volume per day) or as dilution rate (% buffer inflow per hour). Czerkawski and Breckenridge (1977) tested a varying dilution rate ranging from 1.4% to 4.0% per hour. As expected, the pH increased with higher buffer flow whereas the lowest rate resulted in a non-physiological pH for forage-fed animals below 6.5 by using the same buffer composition for all dilution rate variants. The authors concluded that a dilution rate less than 2.08% per hour (0.5 buffer flow rate per day) was too low and did not simulate the conditions in the rumen. However, Klevenhusen et al. (2015), Khiaosa-Ard et al. (2015) and Harder et al. (2015) used a dilution rate below 2.08% and detected no average pH values under 6.5. The buffer itself and buffer rates that were too high must be seen as critical, because it seems to be over buffering the vessels (Orton et al., 2020). Nevertheless, many RUSITEC authors used a higher dilution rate, so that Kajikawa et al. (2003) reported an average of 3.4%/h, which is close to the value presented here (3.2%/h dilution rate; Table 5).

Czerkawski and Breckenridge (1977) found no effect of dilution rate (1.4 to 4%/h) on DM degradability and Carro et al. (1995) detected no effect on disappearance of DM and NDF with dilution rates of 2.3%/h and 3.5%/h. In contrast Martínez et al. (2009) indicated a higher disappearance of DM, NDF and ADF for a high (5.42%/h) compared with a low (3.78%/h) dilution rate (1.3 buffer flow rate per day). Czerkawski and Breckenridge (1977) detected no effect of the dilution rate on methane production and Carro et al. (1995) and Martínez et al. (2009) also found no effect of dilution rate, even though the methane production was strongly affected by pH value. The total VFA production increased by using a high dilution rate of 5.42%/h (Martínez et al., 2009); however, these results contrast with those from Carro et al. (1995), who detected a higher total VFA production with a lower dilution rate (2.3%/h) and Isaacson et al. (1975), who detected no effect on VFA production. Although microbial growth

was not affected by a higher dilution rate, Martínez et al. (2009) suggested that increasing the dilution rate favours changes in microbial populations toward microbes with greater fibrolytic and proteolytic activities. Czerkawski and Breckenridge (1977) and Carro et al. (1995) indicated differences in holotrich protozoa between high and low dilution rate, which may be related to pH sensitivity of holotrich protozoa and the lower pH values at low dilution rates. These differences in results found in RUSITEC studies could be due to the treatment diets, composition of inoculum and different dilution rates (e. g. Carro et al., 1995; Martínez et al., 2009).

Often disregarded factors are the duration of the daily bag changing phase and the washing procedure of the treatment bags (Table 4). Changing and washing takes up to 5 minutes per vessel resulting in 10 to 60 minutes per RUSITEC system depending on handling and number of vessels. This time reduces the effective incubation time of the feed bags but has not been considered yet. Furthermore overflow and gas volumes are measured simultaneously during this period, so that not only the motor is stopped, but the system is open for up to 60 minutes, letting air inflate the headspace of the vessels and the overflow tubes. After closing the system it is therefore recommended to flush tubes and effluent bottles with CO2 or N2. The original washing procedure described by Czerkawski and Breckenridge (1977) includes washing and moderate squeezing of the removed bag, each with 40 mL of artificial saliva. This procedure was only followed by a few authors (Krishna et al., 1986; López et al., 1999; Boguhn et al., 2006; Giraldo et al., 2007; Carro et al., 2009). Others (Table 4) have used artificial saliva quantities of 40, 50, 60, 100 and 140 mL for washing and moderate squeezing (Brice and Morrison, 1991; Akhter et al., 1996; Jasper, 2000; Krause, 2002; Boguhn et al., 2013; Hartinger et al., 2019b). The suspension resulting from squeezing contains loosely solid-adherent microbes as well as small particles and so should be returned to the respective vessel (Czerkawski and Breckenridge, 1977). The washing and closing procedures have often not been described and it is likely that they are not uniformly and consistently performed. Technical details of artificial saliva and the buffer pump of RUSITEC systems should be harmonised to ensure or at least ease the reproducibility of data and comparability of results. To generate a standardisation of buffer handling in RUSITEC trials, it is recommended to exactly follow McDougall's (1948) artificial saliva composition, use a peristaltic pump with a buffer dilution rate of 2.08%/h and wash and moderately squeeze incubation bags during the changing procedure with 50 mL artificial saliva.

Table 5 Information concerning the RUSITEC buffer in 93 published RUSITEC studies covering 111 different technical variants.

Item	N	Mean	SD	Median	Min	Max	Reference no. (see Appendix)
Buffer composition	36	McDougall (1948)					3; 6; 14; 13; 15; 23; 20; 19; 25–27; 34; 36; 38; 43; 48; 49; 51; 54; 60–63; 67; 70; 72; 73; 85; 93
	50	Following McDougall (1948) and additions of further substances					1; 2; 9–12; 14; 16; 17; 23; 22; 28; 30; 35; 37; 39–41; 44–46; 50; 53; 55; 57; 58; 66; 68; 69; 71; 74; 76; 80; 82–84; 86; 87; 89–92
	9	Own or other buffer composition					4; 7; 8; 29; 33; 78; 79
Buffer pump system	25	Peristaltic pump systems					1; 6; 10; 20; 21; 19; 24; 36–38; 45; 46; 48; 52; 54; 58; 68; 75; 77; 78; 91
Buffer supply, mL/day	96	643	165	640	302	1000	1; 2; 4; 6; 5; 7–11; 14; 13; 15–18; 23; 20; 21; 19; 24–30; 32–41; 43–49; 51–68; 70–78; 80–86; 88; 87; 89–93
Buffer flow rate, buffer/vessel volume	90	0.76	0.18	0.75	0.38	1.30	1; 2; 4; 6; 5; 7–11; 14; 13; 15–17; 23; 21; 19; 24–30; 32–41; 43–48; 52–57; 59–67; 70–78; 80–86; 88; 87; 89; 90; 92; 93
Buffer dilution rate, %/h	90	3.17	0.76	3.13	1.59	5.42	1; 2; 4; 6; 5; 7–11; 14; 13; 15–17; 23; 21; 19; 24–30; 32–41; 43–48; 52–67; 70–78; 80–86; 88; 87; 89; 90; 92; 93

3.7. Sample particle size and content for incubation

As comminution by chewing and rumination is missing in the RUSITEC, it is common practice to reduce the particle size of the feeds by grinding or manual chopping. The changes in physical structure influence the availability of nutrients inside the bag and may also lead to high losses of less degradable nutrients contained in small particles. Consequently, bag porosity should be adapted accordingly (Michalet-Doreau and Ould-Bah, 1992). Hildebrand et al. (2011b) found no significant differences in NDF degradation when incubating maize and grass silages ground through 1 mm or 4 mm sieve aperture size. Disappearance of OM and CP, however, was unexpectedly higher with the larger particle size, whereby these sizes are not representative for mechanical chewing. However, Hildebrand et al. (2011b) indicated a higher gas production with smaller particles (1 mm) in the HGT. Particles derived from grinding samples through 1 mm or 3 mm sieve aperture size had only a small effect on the microbial amino acids profile and no effect on OM and fibre digestibility in a continuous-culture system (Rodríguez-Prado et al., 2004). Total VFA concentration was not altered but the proportion of acetate and the acetate to propionate ratio were significantly affected. Hildebrand et al. (2011a) studied the effect of the feed particle size on the synthesis of microbial CP and amino acid profile of microbial fractions. The particle size affected the amino acid profile, and the microbial N (mg/day) in solid-associated microbes was higher with smaller particles sizes in diets with a ratio of 24:76 and 0:100 maize silage to grass silage (Hildebrand et al., 2011a). The results of Hildebrand et al. (2011a, c) suggested an improved fermentation of diets with coarsely milled diets (4 mm and 9 mm) in RUSITEC systems. Between the fine (1 mm) and coarse (4 mm) diets, there was a significant difference in the relative abundance of Prevotella bryantii (Witzig et al., 2010a). Witzig et al. (2010b) detected different microbial community structures with coarsely versus finely ground diets. Furthermore, Witzig et al. (2010b) showed that coarse grinding had a negative effect on the cellulolytic bacterium Ruminococcus albus in comparison to fine grinding of the diet. Both Witzig et al. (2010a, b) and Hildebrand et al. (2011a, b, c) indicated the important role of an adequate environment inside vessels, especially of feed particle size, for the microbial community. The authors indicated that the effect of the feed particle size also depends on the ratio of grass silage to maize silage and is still an important factor for several microorganisms (Witzig et al., 2010a, 2010b; Hildebrand et al., 2011c).

Kajikawa et al. (2003) reported that many researchers used on average 16.4 g DM per bag. In this overview, the range varied from 3 g (Kostyukovsky et al., 1995; Brice and Morrison,

1991) to 20 g (López et al., 1999; Giraldo et al., 2007). These numbers are similar to the results of this study yielding an average of 13.1 g DM and a median of 14.4 g DM per bag (Table 3).

The literature survey supports the need for a standardised comminution by grinding, chopping or cutting of feeds or diets to facilitate comparison of results of across studies of different working groups. In addition, a different amount of incubated DM could influence the amount of end products, e.g. VFA, and perhaps the buffer inflow must be adapted. Furthermore, the volume and particle size of the feed determines the handling inside the food-container and the particle washout losses and must always be taken into account. The particle size should be matched to the pore size of the bags in semi-continuous culture experiments and different conditioning is therefore recommended for coarse forages (particle length of no more than 3 cm through chopping or cutting) and for concentrates (grinding with 1 mm sieve) to generate representative sizes for mechanical chewing (see section 3.3), pore size of incubation bags, bag material, washout losses).

4. Modified semi-continuous simulation technique

Many different types of artificial or simulated fermentation systems have been developed and modified, and applied to a variety of animal species, for instance dog (Kröger, 2009), pig (Bender et al., 2001), deer (Kim et al., 1996), horse (Dill et al., 2007; Kujawa et al., 2020), sheep (Boguhn et al., 2013), goat (Zhao et al., 2013), cattle and bison (Hess et al., 2003; Oss et al., 2016). The suggested basic recommendations for the RUSITEC can also be used for the modified systems of the same technical type, taking into account the specific requirements of the target animal species or section of the gastro-intestinal tract. The COSITEC (colon simulation technique) is used to study the effects on the microbial hindgut metabolism of pigs (Stück et al., 1995; Bender et al., 2001; Mader and Zentek, 2007). Some studies on hindgut metabolism using the COSITEC showed that the system also had a high constancy and reproducibility when started with caecal inocula (Breves et al., 1991; Breves and Dreyer, 1991). Hindgut digestibility of OM, cellulose and hemicellulose in a COSITEC system was similar to the results observed in in situ experiments (Breves and Dreyer, 1991).

Engelmann et al. (2007) used a modified RUSITEC to study the effects of an increasing amount of fructans in equine caecum content. Vosmer et al. (2012) tested the effects of an abrupt change from hay-based feeding to green fodder-based feeding on horses' caecal bacteria, whereas Kuhn (2009) studied the metabolism of antibiotics by microbes in the

hindgut. The caecum simulation technique (CAESITEC) requires caecum content from either slaughtered (Zeyner et al., 2006) or cannulated healthy adult horses (Meyer and Klingeberg-Kraus, 2002). On the basis of animal welfare, alternative methods to obtain starter media must be considered. Dill et al. (2007) compared equine caecum content and faeces as inocula and concluded that caecum content and faeces as inocula provided similar environmental conditions in the CAESITEC.

5. Recommendations for a standardised procedure

- The most important factor before starting RUSITEC procedures is the feeding of the donor animals. A standardised feeding of donor animals, such as in other *in vitro* systems, e.g. the HGT, is recommended – 0.5 to 0.7 hay and 0.3 to 0.5 concentrate on DM basis, 14 days adaptation phase, two meals a day with a minimum between-feeding interval of 8 h.

- The rumen fluid should be taken via vacuum pump before the morning feeding of at least two donor animals, pooled in equal proportions and prepared by filtration through one layer of cheesecloth. Furthermore, the solid material should be harmonised by using more than one sampling location preferably in the central rumen.

- At the start, RUSITEC vessels should be filled with rumen fluid, buffer and deionised water in a ratio of 0.5:0.2:0.1. It is recommended to use vessels with a minimum volume of 800 mL. The second bag should be filled with 80 g of rumen solids.

- The incubation vessel should be packed with two nylon incubation bags with a pore size of 100 µm and of 13 to 16 g DM of the respective diet. Each bag should be incubated for 48 h.

- The buffer composition proposed by McDougall (1948) is recommended. Buffer should be supplied by a peristaltic pump with a buffer flow rate of 0.5 per day (dilution rate of 2.08%/h). Incubation bags removed from the system should be washed once with 50 mL of warm buffer and squeezed moderately.

- Feeds incubated in the RUSITEC must be of appropriate particle size. Forages should be chopped or cut to particle lengths of no more than 3 cm and concentrates should be milled using a 1-mm sieve aperture.

- Moreover, technical details of the RUSITEC apparatus like motor movement, gasbags and tubing material bear opportunities for standardisation, although the effects are considered rather small.

The variability of technical RUSITEC variants and the procedures used to run these systems is exceptionally high, making comparison of results from individual studies extremely difficult. Having evaluated and compared over 90 studies using RUSITEC systems, we strongly recommend standardising technical details and methodical aspects.

CRediT authorship contribution statement

Jan-Helge Deitmers: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Nina Gresner:** Investigation, Writing – review & editing. **Karl-Heinz Südekum:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing.

Conflict of interest statement

All authors declare no competing interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2022.115325.

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Appendix.

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CHAPTER 4

Comparison of ruminal fermentation characteristics of two common forages using a coupled in vivo-in situ approach and the in vitro rumen simulation technique RUSITEC

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A B S T R A C T

The increasing demand for a reduction of animal experiments when studying rumen fermentation has led to the development of various *in vitro* techniques, such as the rumen-simulation technique (RUSITEC) system that is prominently applied in European ruminant research. Yet, comparability with the *in vivo* situation is rather less explored with a sparse data basis. Therefore, the present study aimed to directly compare the fermentation characteristics and degradability of two common forages, i.e., grass silage (GS) and maize silage (MS), by parallel application of a coupled *in vivo*-*in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system. Both forages were incubated in the RUSITEC system as well as fed to rumen-cannulated sheep in six independent runs of 20 days in total with 14 days of adaptation and 6 days of sampling. The degradability coefficients of dry matter, organic matter and acid detergent fibre were affected by the method (each $P < 0.05$), while neutral detergent fibre (aNDFom) degradability was not different between RUSITEC and *in situ* measurements ($P = 0.10$). Likewise, Pearson correlation coefficients confirmed the comparability of *in vitro* and *in situ* values for aNDFom degradability, being 0.54 ($P = 0.04$) and 0.78 ($P = 0.02$) for GS and MS, respectively. Regarding the fermentation profile, total volatile fatty acid (VFA) concentrations were much higher *in vitro* than *in vivo* ($P < 0.01$), likely due to the missing absorptive capacity of the RUSITEC system. A comparison of absolute fermentation values between methods appears not feasible. However, the order of individual VFA proportions was similar between *in vivo* and *in vitro* and the correlations for both total and individual VFA further supported this congruency, especially for MS. The *in vitro* data appeared well comparable to the data from the coupled *in vivo*-*in situ* approach, especially for MS, with a high reproducibility in both methods. Therefore, the RUSITEC system may represent a sufficient replacement for laborious *in vivo* and *in situ* measurements when assessing nutrient degradability and general fermentation characteristics of feedstuffs. Adjustments in *in situ* incubation times as well as the frequently requested standardization of the operation of the RUSITEC system may further increase the significance of this *in vitro* method in the future. Likewise, further research on diurnal fermentation patterns is encouraged to substantiate the present findings.

Keywords: *in sacco;* replacement method; ruminant; silage

Abbreviations: ADF, acid detergent fibre; ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed with a heat-stable amylase and expressed exclusive of residual ash; ADL, acid detergent lignin; DM, dry matter; GS, grass silage; MS, maize silage; NDF, neutral detergent fibre; RUSITEC, rumen simulation technique; WSC, water-soluble carbohydrates; VFA, volatile fatty acid.

1. Introduction

Due to ethical as well as workload reasons, various *in vitro* techniques that allow the reduction of animal experiments to study rumen fermentation have been developed and continuously improved over the last decades (GfE, 2017; Deitmers et al., 2022). However, comparability to *in vivo* results constitutes a prerequisite for the meaningfulness of any *in vitro* system, which yet often remains to be evaluated. The *in vitro* methods developed in the past comprise a large variety of systems with different purposes, including short-term batch culture systems such as the Hohenheim gas production test as well as elaborated continuous or semi-continuous culture systems that enable the long-term simulation of the rumen (Slyter et al., 1964; Weller and Pilgrim, 1974; Hoover et al., 1976; Czerkawski and Breckenridge, 1977). In contrast to *in vivo* experiments, compared to *in vivo* feeding trials, *in vitro* systems facilitate the investigation of a large number of treatments in a relatively short period of time. In particular complex long-term *in vitro* systems have received broader attention as they provide a closer and more realistic simulation of the *in vivo* situation that may in turn improve the significance of results obtained and their transferability to the *in vivo* situation in the rumen.

Among these complex long-term *in vitro* systems, the rumen simulation technique (RUSITEC) of Czerkawski and Breckenridge (1977) represents a frequently used and well accepted method for pursuing research questions on rumen fermentation, especially in Europe (e.g., Boguhn et al., 2006; Hildebrand et al., 2011a, 2011b; Hartinger et al., 2019a). However, studies directly comparing *in vivo* vs. *in vitro*, i.e. to the RUSITEC system, are yet sparse and the few available studies used either pooled inoculum for the *in vitro* incubation or varying numbers of animals for the *in vivo* and *in vitro* trials (Carro et al., 2009; Martínez et al., 2010). A direct animal-individual comparison with the RUSITEC system, which not only provides a comparison of *in vivo* vs. *in vitro* but also information on the variation of results, is lacking so far. Consequently, there is a clear demand for a reliable data basis and our objective was to directly compare the fermentation characteristics and degradability of two common forages by parallel application of a coupled *in vivo-in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system. We hypothesized that the *in vitro* RUSITEC system provides similar results in terms of fermentation characteristics and forage degradability as obtained in sheep. Further, as the experiment was designed as an animal-individual comparison with the RUSITEC system, we also expected a low variation within sheep and RUSITEC vessels.

2. Materials and methods

The experimental animals were kept according to the German Animal Welfare legislation at the Institute of Animal Science of the Faculty of Agriculture, University of Bonn in Germany. All experimental procedures were conducted in accordance with the German guidelines for animal welfare and were approved by the Animal Care Committee of the State of North Rhine-Westphalia in Germany (file number 81-02.05.40.18.008).

2.1. Experimental diets

Maize silage (MS) and grass silage (GS; pure stand of Italian ryegrass, *Lolium multiflorum*) used in this study originated from bunker silos or round bales of the Campus Frankenforst of the Faculty of Agriculture, University of Bonn (Königswinter, Germany; 7° 12′ 22″ E; 50° 42′ 49″ N). After an ensiling period of 100 days and opening of bunker silos and round bales, the the material was re-ensiled in 120 L plastic barrels for better handling during the trial period and to ensure the provision of unspoiled silage. The chemical composition of both forages is presented in Table 1. Due to a deficiency in crude protein concentration, the MS was supplemented with urea by addition of 15 g urea-N into the ration as well as by inclusion of 0.535 mg urea-N per ml into the buffer solution for the sheep and the RUSITEC system, respectively. Therefore, both forages were provided isonitrogenously.

Table 1 Chemical composition of grass silage and maize silages (g/kg dry matter unless otherwise stated).

	Grass silage	Maize silage
Dry Matter, g/kg	357	377
Organic Matter	877	967
Crude protein	125	67.2
Ether extract	28.6	32.1
aNDFom[1]	469	325
ADFom[2]	312	185
ADL[3]	28.3	19.6
Starch	not analysed	451
WSC[4]	94.2	11.2
pH	4.37	3.64
Lactic acid	70.0	76.4
Acetic acid	20.5	13.4
Ammonia-N, g/kg N	125	167

[1]aNDFom = Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.

[2]ADFom = Acid detergent fibre expressed exclusive of residual ash.

[3]ADL = Acid detergent lignin.

[4]WSC = Water-soluble carbohydrates.

2.2. Coupled in vivo-in situ approach

Six Merino- and Rhönschaf × Suffolk crossbreed sheep (mean body weight 85.1 ± 17.3 kg) fitted with permanent ruminal cannulas were used for the experiment and were fed at 120% of maintenance energy requirements. Drinking water were continuously available during the complete experiment. The experiment comprised six independent runs of 20 days in total with 14 days of adaptation period and 6 days of sampling period. Consequently, GS and MS were fed in three runs each in an alternating mode. In each run, GS or MS supplemented only with NaCl were offered to three sheep each twice a day for one hour (0730 h and 1930 h). The individual feed intake was calculated by weighing the feed before and after feeding for each animal.

During the complete experimental period, 10 ml of ruminal fluid was collected via the standardized procedure of Tafaj et al. (2001) from each sheep before the morning feeding to immediately measure the rumen pH via potentiometry (BlueLine 14 pH, SI Analytics, Mainz, Germany, and pH 315i, WTW, Weilheim, Germany). During the sampling period (experimental days 15 to 20), additional ruminal fluid samples were collected directly before the morning feeding, for VFA and ammonia-N analyses and directly stored at -18°C. On experimental days 16 and 18, *in situ* incubations were performed according to standardized protocol of Kirchhof (2007) and Südekum (2005). Briefly, three nylon bags (100 mm × 200 mm, 50 µm pore size, ANKOM Technology, Macedon, NY, USA) containing approximately 5 g DM of the respective silage were incubated for 48 h in the rumen of each sheep. Before insertion into the rumen, bags were incubated in a water bath at 39°C for 15 min and then placed below the fibre mat in the rumen and fixed with a nylon cord to avoid displacement. After removal from the rumen after 48 h, bags were rinsed with cold water and stored at -18°C until further analysis.

2.3. In vitro RUSITEC system

In order to allow a comparison with the coupled *in vivo-in situ* approach, the same silage fed to sheep during one respective run was simultaneously incubated in the RUSITEC system. Therefore, again six independent runs of 20 days in total with 14 days adaptation period and 6 days sampling period were performed simultaneously in the RUSITEC system (Czerkawski and Breckenridge, 1977). Comprehensive information on setup, implementation and daily routine of the six-vessel RUSITEC system are given in detail in Hartinger et al. (2019a). In brief, each sheep was assigned to one vessel, and the liquid and solid inoculum was obtained individually from each sheep on day 0 before the morning feeding, strained through two layers of cheesecloth and immediately transferred to the corresponding vessels within 30 min.

Each day, 14.6 g DM MS (chopped to 6-8 mm particle length) or 16.8 g DM GS (chopped to 5 cm particle length) filled in nylon bags (140 × 80 mm, 1000 µm pore size, Klein & Wieler oHG, Königswinter, Germany) were incubated in the vessels. The nylon bags were incubated for 48 h and washed with 50 mL pre-warmed buffer solution after removal. The liquid effluent was collected in cooled glass containers and volumes were measured daily.

During the sampling period, analogous to the coupled *in vivo-in situ* approach, 10 mL of fluid were taken via a 3-way-valve from each vessel prior to the nylon bag exchange. Then, pH was directly measured by potentiometry (BlueLine 14 pH, SI Analytics, Mainz, Germany, and pH 315i, WTW, Weilheim, Germany) and aliquots for VFA and ammonia-N analysis were stored at -18°C. Likewise, nylon bags with feed residuals of days 16 and 18 were stored at -18°C until further analysis. Again, pH was measured immediately and samples for VFA and ammonia-N were frozen at -18°C until further analysis.

2.4. Sample analyses

The silages and feedstuff residues of the *in situ* and RUSITEC incubations from days 16 and 18 were freeze-dried (P18K-E- 6, Piatkowski, Petershausen, Germany) and milled through a 3 mm screen (RETSCH SM 100, Retsch, Haan, Germany). Subsequently, samples were analysed according to German Handbook of Agricultural Experimental and Analytical Methods (VDLUFA, 2012). The DM concentration was determined according to method 3.1 and the values were subsequently corrected for the loss of volatiles during drying using the equation of Weißbach and Strubelt (2008a) for GS and Weißbach and Strubelt (2008b) for MS. The ash and ether extract concentrations as well as the concentrations of neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash (aNDFom), acid detergent fibre expressed exclusive of residual ash (ADFom) and acid detergent lignin (ADL) were analyzed according to methods 5.1.2, 8.1, 6.5.1, 6.5.2 and 6.5.3, respectively. For the calculation of OM, the ash content was subtracted from the DM of the feed residues.

Furthermore, pH as well as concentrations of water-soluble carbohydrates, lactic acid, acetic acid and ammonia-N were determined in cold-water extracts prepared from 50 g of fresh GS and MS as outlined in Hartinger et al. (2019b). The apparent disappearance of DM, OM, aNDFom and ADFom during *in situ* and *in vitro* incubation was calculated from the difference between the amount of DM, OM, aNDFom, ADFom before the incubation and the amount recovered in 48 h samples.

The analysis of VFA (i.e. acetate, propionate, n-butyrate, n-valerate, n-caproate, isobutyrate and isovalerate) was performed via gas chromatography (GC-2014, Shimadzu, Duisburg, Germany) as described in Hartinger et al. (2019a). Briefly, samples were thawed on ice and centrifuged at 20,000 × g for 15 min. The supernatant was transferred into a new tube, mixed with 100 µL of internal standard (formic acid) and centrifuged again. Subsequently, VFA concentrations were determined in the clear supernatant. The ammonia-N concentration was determined by Kjeldahl method via automated distillation (Vapodest 50s carousel, Gerhardt, Königswinter, Germany) according to Keay and Menage (1969).

2.5. Statistical analysis

One sheep was excluded from the trial due to impaired health condition before the experimental start and both, the *in vivo-in situ* and the *in vitro* data set, were therefore reduced. Data were checked for normal distribution using the Shapiro-Wilk test of the UNIVARIATE procedure in SAS v9.4 (SAS Institute Inc., Cary, NC, USA). Subsequently, datasets were statistically analysed using the repeated-measurements MIXED model procedure in SAS v9.4 to test for the effects of method and forage, the following model was used:

$$Y_{ijkl} = \mu + M_i + F_j + L_k + T_l + (M \times F)_{ij} + e_{ijkl}$$

where Y is the observed response, µ is the overall mean, M_i is the fixed effect of method (*in vivo-in situ* or *in vitro*), F_j is the fixed effect of forage (MS or GS), L_k is the random effect of experimental run, T_l is the random effect of vessel or animal and e_{ijkl} is the residual error. If significant interaction effects were observed, differences between forages within method were analysed using the post-hoc Tukey test. Significance was defined at $P < 0.05$ and a trend at $0.05 \leq P \leq 0.1$.

Additionally, Pearson correlation coefficients between *in vivo-in situ* and *in vitro* data sets were calculated using proc CORR in SAS v9.4 (SAS Institute Inc., USA). The coefficients were ranked according to a scale presented by Akoglu (2018) to enable a uniform interpretation of their strengths. Strong and moderate correlations are indicated by absolute values ranging from 0.7 to 0.9 and 0.4 to 0.6, respectively.

3. Results

3.1. Fermentation profile

As presented in Table 2, incubation of GS in the RUSITEC system resulted in a lower pH than when fed to sheep, while the *in vivo* pH was lower with MS than with GS. This was not the case *in vitro* ($P < 0.001$), which is indicated by a significant method x silage interaction effect. The ammonia-N concentration was higher *in vitro* than *in vivo* ($P < 0.001$) and also affected by forage with higher ammonia-N concentrations with MS than with GS ($P < 0.001$).

We observed an interaction of method and silage for n-valerate and n-caproate (each $P < 0.001$) with higher proportions *in vitro* during GS incubation compared to MS incubation, whereas no difference between GS and MS feeding was present *in vivo*. The method showed an effect and revealed higher proportions *in vitro* than *in vivo* for total VFA as well as all individual VFA (each $P < 0.001$), except propionate ($P = 0.022$) and isobutyrate ($P = 0.496$). Moreover, silage type influenced the proportions of propionate and n-caproate (each $P < 0.001$) were propionate proportions were higher with MS than with GS, and also total VFA tended to be higher with MS than with GS ($P = 0.084$).

Table 2 Daily fermentation characteristics in *in vitro* RUSITEC fermenters and *in vivo* sheep rumen with grass silage (GS) or maize silage (MS).

	In vitro		*In vivo*			P-value		
	GS	MS	GS	MS	SEM	Method	Silage	Method × silage
pH	6.59^{B}	6.58	6.82^{aA}	6.57^{b}	0.032	<0.001	<0.001	<0.001
Ammonia-N, mg/L	140.2	260.9	91.9	187.8	12.30	<0.001	<0.001	0.239
Volatile fatty acids								
Total, mmol/L	112.2	113.8	61.5	69.2	4.90	<0.001	0.084	0.233
Proportion of total (mol/mol)								
Acetate	0.510^{aA}	0.544^{b}	0.690^{aB}	0.617^{b}	0.009	<0.001	0.037	<0.001
Propionate	0.182	0.206	0.194	0.246	0.010	0.002	<0.001	0.077
n-Butyrate	0.131	0.152	0.078	0.090	0.008	<0.001	0.028	0.532
n-Valerate	0.051^{aA}	0.029^{bA}	0.012^{B}	0.014^{B}	0.003	<0.001	0.001	<0.001
n-Caproate	0.037^{aA}	0.013^{b}	0.005^{A}	0.006	0.003	<0.001	<0.001	<0.001
Isobutyrate	0.009	0.005	0.008	0.009	0.002	0.496	0.401	0.312
Isovalerate	0.080^{aA}	0.050^{bA}	0.012^{B}	0.016^{B}	0.005	<0.001	0.013	0.001
Acetate:propionate	2.94	2.67	3.65	2.56	0.20	0.471	<0.001	0.075

Capitalized superscript letters indicate differences between methods within the same forage ($P < 0.05$).

Lowercase superscript letters indicate differences between forages within the same method ($P < 0.05$).

3.2. Degradability of DM, OM, aNDFom and ADFom

Table 3 illustrates the *in vitro* and *in situ* rumen degradability coefficients for DM and OM as well as the fibre fractions aNDFom and ADFom. The data revealed interactions of method and silage for all variables. Thereby, DM ($P = 0.030$) and OM ($P = 0.015$) degradability coefficients were lower in GS than in MS for the *in vitro* incubation, but not differing for the *in situ* approach. In contrast, aNDFom and ADFom degradability coefficients (both $P < 0.001$) were lower in MS than in GS for the *in situ* incubation, but not differing for the RUSITEC data.

Regarding main effects, applied method affected the degradability coefficients of DM ($P < 0.001$), OM ($P < 0.001$) and ADFom ($P < 0.043$) with lower values *in vitro* than *in situ*, whereas aNDFom degradability was not different between the incubation techniques. In addition, silage type had an impact on the fibre degradabilities (both $P < 0.001$), being lower for MS than for GS.

Table 3 Degradability coefficients of grass silage (GS) and maize silage (MS) during *in situ* or *in vitro* incubation for 48 h.

	In vitro		*In situ*			P-values		
	GS	MS	GS	MS	SEM	Method	Silage	Method × silage
Dry matter	0.593[b]	0.613[a]	0.729	0.708	0.011	<0.001	0.916	0.030
Organic matter	0.581[b]	0.617[a]	0.716	0.705	0.011	<0.001	0.209	0.015
aNDFom[1]	0.400	0.353	0.561[a]	0.251[b]	0.019	0.104	<0.001	<0.001
ADFom[2]	0.390	0.343	0.596[a]	0.216[b]	0.022	0.043	<0.001	<0.001

[1]aNDFom = Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.

[2]ADFom = Acid detergent fibre expressed exclusive of residual ash.

Lowercase superscript letters indicate differences between forages within the same method ($P < 0.05$).

3.2. Correlation analysis

All correlations coefficients and associated P-values are presented in Table 4. The aNDFom degradability between *in situ* and *in vitro* measurements revealed a moderate (r = 0.54; P = 0.04) and strong (r = 0.78; P = 0.02) correlation for GS and MS, respectively. For MS, a strong correlation between *in situ* and *in vitro* data was also present for ADFom degradability, i.e. r = 0.85 and P=0.01. Similar to aNDFom degradability, total VFA concentration was moderately (r = 0.55; P = 0.03) and highly (0.74; P = 0.02) correlated for GS and MS, respectively. At the individual VFA level, *in vivo* and *in vitro* data showed moderate correlations of 0.62 (P = 0.01) for propionate as well as of 0.57 (P = 0.03) for n-butyrate concentrations. Additionally, caproate tended to be moderately correlated between *in vivo* and *in vitro* data, i.e. r = 0.48 and P = 0.07. Regarding MS, concentrations of acetate and propionate were strongly correlated between *in vivo* and *in vitro* with 0.72 (P = 0.03) and 0.75 (P = 0.02), respectively.

Table 4 Pearson correlation coefficients for variables measured via the coupled *in vivo-in situ* approach and *in vitro* for grass silage and maize silage separately. Significant correlations (P<0.05) are marked in bold print.

Variable	Grass silage		Maize silage	
	Coefficient	P-value	Coefficient	P-value
DM degradability	0.33	0.23	0.56	0.12
OM degradability	0.41	0.13	0.55	0.12
aNDFom degradability	**0.54**	**0.04**	**0.78**	**0.02**
ADFom degradability	0.14	0.61	**0.85**	**0.01**
Ammonia-N	0.42	0.12	-0.54	0.13
Total VFA	**0.55**	**0.03**	**0.74**	**0.02**
Acetate	0.41	0.13	**0.72**	**0.03**
Propionate	**0.62**	**0.01**	**0.75**	**0.02**
n-Butyrate	**0.57**	**0.03**	0.45	0.22
n-Valerate	-0.27	0.32	0.31	0.42
n-Caproate	0.48	0.07	0.47	0.21
Isobutyrate	-0.04	0.87	-0.37	0.33
Isovalerate	0.13	0.64	0.25	0.52
pH liquid phase	-0.01	0.87	0.08	0.28

4. Discussion

The present study provides a direct comparison of the fermentation characteristics and degradability of two common forages by parallel application of a coupled *in vivo*-*in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system. Thereby, the hypothesis was set that the *in vitro* RUSITEC system provides similar results in terms of fermentation characteristics and forage degradability as obtained in sheep rumen. Secondly, we expected a low variation within sheep and RUSITEC vessels in the animal-individual comparison.

The degradability data revealed an impact of methodology on degradabilities of DM, OM and ADFom measured by *in situ* or *in vitro* procedure. However, no difference was found for the aNDFom fraction and our correlation analysis indeed showed a moderate and even strong correlation for aNDFom degradability of GS and MS, respectively. Consequently, it appears that RUSITEC-derived degradability coefficients can be sufficient proxies for *in situ* measurements, representing a much less laborious and more cost-effective strategy to assess ruminal degradability of total structural carbohydrates, i.e., aNDFom comprising hemicelluloses, cellulose and lignin. Comparing fibre degradabilities obtained *in situ* to those received from a short-term *in vitro* system, Trujillo et al. (2010) also observed a consistent ranking of feedstuffs with both methods, which supports the general feasibility of reducing *in situ* measurements by *in vitro* procedures for assessing ruminal fibre degradability. In addition, *in vitro* ADFom degradability of MS was strongly correlated with the *in situ* measurements, therefore indicating that comparability of methods may depend on the forage type, which became also apparent from the significant interactions found for degradability coefficients.

In this context, we believe that the overall satisfying consistency in *in situ* and *in vitro* fibre degradability coefficients may be further improved by adapting the incubation time of nylon bags into the rumen. The 48-h incubation time, which was chosen for both methods in the present study, is a fixed figure in the RUSITEC system, but can be flexibly designed with the *in situ* method. Thereby, an adaptation of the *in situ* incubation time may translate into improved concordance between *in situ* and *in vitro* degradabilities for fibre fractions but also DM, OM and other nutrients. In fact, a reduction of incubation time should more closely resemble the true *in vivo* rumen scenario, where 48 h retention time of forages is rather unlikely, especially in the case of MS or high feed intake levels (Krämer et al., 2013). A follow-up comparison study with several time points for the *in situ* incubations, analogous to

the determination of ruminal degradation kinetics (Kirchhof, 2007), may then provide information on a suitable incubation time for a specific comparison with RUSITEC data. For a comparison of RUSITEC data across studies, however, a standard incubation time is required (Deitmers et al. 2022).

Regarding ruminal fermentation, concentrations of total VFA were on a substantially higher level *in vitro* than *in vivo*, which can be explained by the lack of any absorptive capacity in the RUSITEC system (Carro et al., 2009). The fermentation end products are only removed via the effluent flow, i.e. simulating the outflow to the lower digestive tract, whereas direct absorption constitutes a dominant mechanism of VFA removal in the rumen (Aschenbach et al., 2011). Likewise, this may also serve as an explanation for the higher ammonia-N concentrations found *in vitro* compared to *in vivo*. Notably, the high ammonia-N concentrations observed with MS both in sheep and in the RUSITEC system should derive from the urea supplementation as MS is typically low in nitrogen compounds and therefore explains the discrepancy with GS richer in crude protein (Boguhn et al., 2013). Consequently, a direct comparison of absolute fermentation values originating from *in vivo* and the RUSITEC system appears not feasible. In this context, we also observed an impact of method on proportions of individual VFA. Therefore, not only absolute but also relative VFA concentrations varied between *in vitro* and *in vivo*, which deserves attention in future studies. Presumably, differences in the microbial community and its metabolic activity could be causative (Martínez et al. 2010). Nevertheless, the order of individual VFA proportions was similar between *in vivo* and *in vitro* and the calculated correlations for both total and major individual VFA further supported this congruency, which was especially true for MS. Similarly, Yanza et al. (2022) observed a consistent effect of *Coleus amboinicus* Lour polyphenols on the final products of ruminal fermentation in both *in vitro* and *in vivo* experiments.

The RUSITEC system did not reproduce the diurnal pH pattern and so appeared less suitable for replacing *in vivo* measurements. This *in vitro* system possesses a high buffering capacity when run with the typically used buffer solution (Khiaosa-Ard et al., 2020; Deitmers et al., 2022). Coupled with its missing absorption function that led to higher VFA concentrations in the fermenters (Carro et al., 2009), it may explain the absence of any pH drop in the RUSITEC after feedbag exchange that, however, was clearly observed 2-4 h after feeding MS to the sheep.

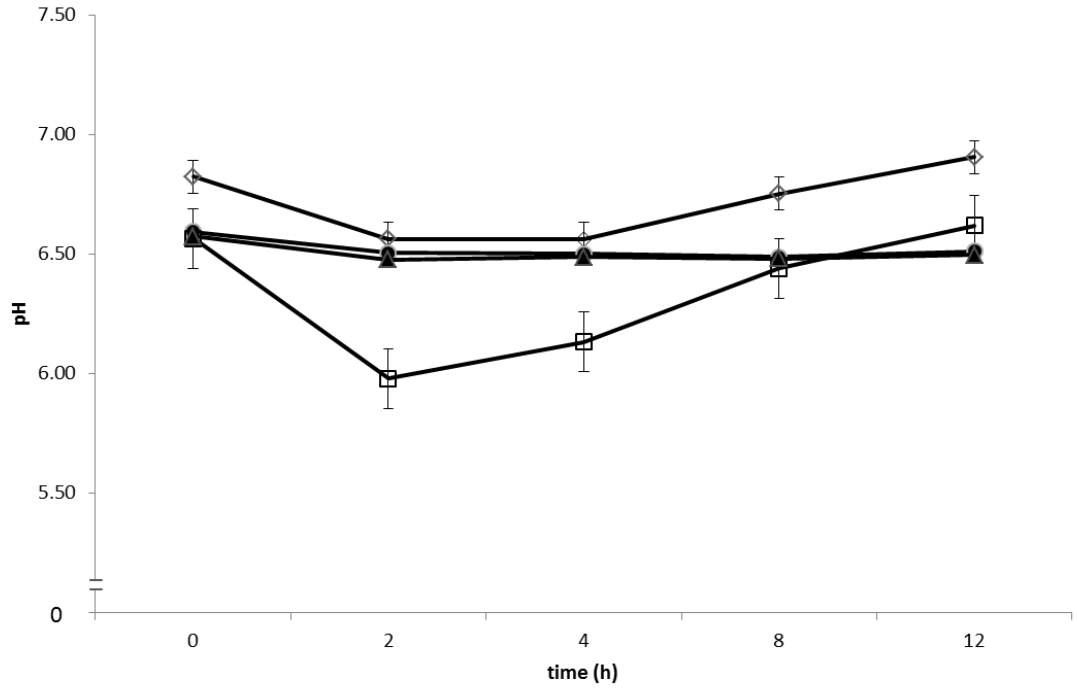

Figure 1 Diurnal pH pattern in the rumen of sheep and RUSITEC fermenter receiving the same silage, i.e., maize silage and grass silage. Sheep were fed at 0 h and 12 h and RUSITEC fermenters were incubated with fresh forage once daily at 0 h. Error bars represent SEM. (▲fermenter incubated with maize silage, ● fermenter incubated with grass silage, □ sheep fed maize silage, ◊ sheep fed grass silage).

Moreover, the diurnal pH patterns were similar with both forages in the RUSITEC, which was not the case for the *in vivo* situation and due to the distinct chemical composition of GS and MS, predominantly the starch content, also not expected. Thus, our hypothesis had to be rejected since the acidotic impact of the present MS was blurred *in vitro* and eventually revealed this limitation of the RUSITEC system – at least for starch-rich feedstuffs.

Similarly, Hildebrand et al. (2011a) obtained uniform pH patterns when incubating GS- and MS-based diets in the RUSITEC, suggesting that this limitation of the RUSITEC is not only restricted to the incubation of pure forage diets but seems to be a general feature of this *in vitro* system. Yet, adaptations in buffer solution and/or infusion rate may enable a better accordance of *in vivo* fermentation patterns with those from the RUSITEC system, which then would further increase the significance of *in vitro* systems and should be pursued in future research. However, despite all efforts to simulate *in vivo* conditions as closely as possible, a meta-analysis by Hristov et al. (2012) revealed that RUSITEC and continuous culture systems are characterized by lower total volatile fatty acid (VFA) and acetate concentrations, low counts or lack of ruminal protozoa, and lower organic matter and NDF ruminal degradability. In addition, the variability of data obtained by using RUSITEC and continuous culture

systems is much higher than for *in vivo* experiments (Hristov et al., 2012).Worthy of remark, however, the repeatability of our findings with the coupled *in vivo*-*in situ* approach in rumen-cannulated sheep as well as with the *in vitro* RUSITEC system was indeed given, as evidenced by the hypothesized low variation within sheep and RUSITEC vessels, respectively.

5. Conclusions

Our study investigated the comparability of fermentation characteristics and degradability of GS and MS in a coupled *in vivo*-*in situ* approach and *in vitro* RUSITEC system. We observed a high congruence of degradability values of both methods, especially the aNDFom degradability showed high similarity. Still, a forage-dependent impact was indicated by higher correlations coefficients found for MS than for GS. The comparability of VFA profiles between the approaches was not given, likely due to a lack of absorption capacity in the RUSITEC system. Still, we observed strong correlations for total VFA and the predominant individual VFA between *in vitro* and *in vivo*. Therefore, the *in vitro* data seemed well comparable to the data derived from the coupled *in vivo*-*in situ* approach.

The RUSITEC system constituted a sufficient replacement for more laborious *in vivo* and *in situ* measurements when investigating nutrient degradability and ranking general fermentation characteristics of feedstuffs. For the purpose of studying diurnal fermentation patterns, however, the RUSITEC system may have limitations in resembling the *in vivo* situation that need to be acknowledged. However, further investigations on diurnal fermentation patterns are requested to support the present results and extent the knowledge.

Declaration of Competing Interest

The authors have no conflict of interest.

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CHAPTER 5

General discussion and conclusions

In this section the key points of the two main chapters of the thesis are discussed, namely the review (Chapter 3) and the rumen simulation technique (RUSITEC) trials (Chapter 4) which both aimed at contributing to a standardisation of the RUSITEC system. This system from the 1970s (Czerkawski and Breckenridge, 1977) is currently receiving renewed attention, mainly because of the increased public awareness of animal welfare and the aim of animal nutrition research to decrease the number of cannulated animals used for experimental purposes (GfE, 2017) leading to further developments according to the 3-R-concept of Russell und Burch (1959) which has consistently received attention including considerations on interactions among the three Rs (de Boo et al., 2005) and suggestions for an improved clarity in definition and purpose of the concept (Tannenbaum and Bennett, 2015).

After focusing on the comparability of RUSITEC and *in vivo* data, the general discussion will also consider the debate concerning the comparability of studies applying different layouts of the RUSITEC system. Finally, the conclusion finishes with a recommendation for standardisation of the RUSITEC procedure.

Ruminal simulation technique and *in vivo* studies

The main target of animal research, especially concerning nutrition and animal feeding experiments, is the live animal. In a variety of experiments in laboratories around the world, the results of animal trials, i.e. *in vivo*, are important factors for calibration and validation of laboratory methods such as RUSITEC. Generally, for the RUSITEC system, the question of the comparability of RUSITEC with *in vivo* trial conditions and results arises. Because the rumen is a very complex and vivid ecosystem, it is difficult to study its function under strictly controlled conditions reflecting a steady-state behaviour. For example, the handling of rumen cannula and it needs variables customised rumen cannula for each research question because every opening enables the inflow of oxygen. Additionally, studies with animals are both expensive and time-consuming, and simulating ruminal fermentation can help to reduce the variability of trial effects while simultaneously reducing the high costs. However, the comparability also depends on the design of the trials, because it is possible to use high (ruminal) flow rates or unusual feed ingredients up to higher toxic levels of feed additives in *in vitro* studies (Hristov et al., 2012).

In contrast to *in vivo* experiments, continuous culture *in vitro* systems facilitate the investigation of a large number of treatments in a relatively short period. Other than simpler systems such as batch cultures, complex continuous culture systems simulate the removal of fermentation end products and maintain a stable fermentation for a longer period (Czerkawski and Breckenridge, 1977) resulting in a closer simulation of the *in vivo* situation. When comparing continuous culture data with those from *in vivo* studies, substantial differences between these two approaches must be considered such as the passage rates of fluid and solid phases, feed intake per rumen volume, and the accumulation of fermentation end products because of a missing rumen wall in continuous culture systems (Stewart et al., 1961; Czerkawski, 1984) . Logically a direct comparison cannot be made, and thus only data with the same units (e.g., proportions and concentrations) can be considered.

Despite all efforts to simulate *in vivo* conditions as closely as possible, a meta-analysis by Hristov et al., (2012) revealed that RUSITEC and continuous culture systems are characterized by lower total volatile fatty acid (VFA) and acetate concentrations, low counts or even a complete lack of ruminal protozoa, and lower ruminal digestibility of organic matter (OM) and neutral detergent fibre (NDF). Moreover, the variability of the data obtained from RUSITEC and continuous culture systems was much higher than was observed *in vivo* (Hristov et al., 2012). Part of this variation is likely explained by the variability or low degree of standardisation of the technical layout of semi-continuous and continuous culture systems unlike the *in situ* method, which has undergone a significant process of standardisation (e.g., Südekum, 2005) resulting in strictly standardised protocols, most recently in Germany (GfE, 2022). Although the diet fed to the donor animal is more important than the animal species itself (Boguhn et al., 2013), both should be considered when RUSITEC and *in vivo* experiments are compared. The lack of information and harmonisation of feeding donor animals is also considered and discussed in detail in Chapter 3, which shows that the variation in feeds or diets, coupled with the, often insufficient, documentation of diets in studies, makes it difficult to compare data.

However, if RUSITEC users would follow a more strictly standardised protocol, the system would be a promising method for standardised feed evaluation. It could then offer a relatively stable, uniform environment and allow complete control of conditions, e.g., ambient temperature and buffer infusion rate, which altogether would lead to enhanced repeatability of results. Similarly, Hristov et al., (2012) also concluded that the variability was much greater for continuous culture systems including RUSITEC compared with *in vivo* experimental data,

which could be due to the variability of designs of fermenters, variability in the ruminal inoculum, and perhaps more extreme experimental treatments than those *in vivo*. Furthermore, the results of Chapter 4 show that, for certain variables, the RUSITEC data seemed well comparable to data derived from a coupled *in vivo*-*in situ* approach. The RUSITEC system constituted a sufficient replacement for more laborious *in vivo* and *in situ* measurements related to ruminal nutrient degradability and the ranking of general fermentation characteristics of feedstuffs. To study diurnal fermentation patterns, however, the RUSITEC system may have limitations in resembling the *in vivo* situation that needs to be acknowledged and justifies further investigation.

Ruminal simulation technique and *in vitro* systems

Most of the research on rumen metabolism is conducted on ruminally cannulated animals, but it is difficult to study in a very complex ecosystem under strictly controlled conditions. Studies with *in vitro* methods reduce the time required and variability of the results in case of replications and can additional avoid high costs. A further advantage of *in vitro* techniques is the possibility to test higher and, in some cases, potentially toxic, levels of feed additives (Hristov et al., 2012; Klevenhusen et al., 2021). In contrast to *in vivo* experiments, continuous culture *in vitro* systems allow the investigation of a large number of treatments in a relatively short period. Simple *in vitro* systems including batch cultures, i.e., without constant inflow and no outflow, such as the Hohenheim gas test (Menke et al., 1979) are hampered by the accumulation of metabolites, which may already inhibit metabolic processes and the viability of rumen microbes after a few hours.

In addition, complex continuous culture systems simulate the removal of fermentation end products and maintain a stable fermentation for a longer period (Czerkawski and Breckenridge, 1977) resulting in a closer simulation of the *in vivo* situation. In addition to the RUSITEC, other artificial rumen systems would be suitable for making a comparison. Generally, artificial rumen systems offer possibilities to evaluate the effects of diet composition (Hildebrand et al., 2011), feed additives on the ruminal disappearance rate of feed (Díaz et al., 2017), microbial crude protein synthesis (Carro and Miller, 1999; Russi et al., 2002; Wischer et al., 2013), microbial community structure (Soto et al., 2013), and ruminal fermentation (Carro et al., 2009). In contrast to the positive aspects, a meta-analysis by Hristov et al. (2012) revealed that RUSITEC and continuous culture systems are not only characterised by comparable data collection (e.g. total VFA and acetate concentrations, ruminal protozoa, and digestibility of OM and NDF).

Future perspectives

A continual development of the RUSITEC system can provide a valuable contribution to *in vitro* research. However, this requires more than a simple standardisation of the technique and necessitates adjustments of the handling. Chapter 3 clearly illustrates the diverse ways in which the RUSITEC system is used, supplemented by the approach, which provides suggestions for how to standardise the system can faciliate the development, use, and acceptance of RUSITEC data in *in vitro* ruminal research. A future perspective could be combining *in vitro* systems in a way that fluids from *in vitro* systems serve as a source of inocula for other, simpler systems. Previously, Barbi et al. (1993) used vessel or effluent fluids from RUSITEC for a simple *in vitro* digestion system. In a small trial without statistical evaluation, we used RUSITEC vessel fluid from a fermented maize silage (MS) and a grass silage (GS) feed at the end of the experiment detailed in Chapter 4 as inoculum in a Hohenheim gas test (HGT), and compared the 24-h gas production with the results of a HGT following the standard procedure (VDLUFA, 2012). The vessel fluid was based on the respective silage (GS or MS) as sole feed. The RUSITEC was started with inoculum from sheep (Chapter 4), whereas the standard HGT was started with inoculum from steers. The results of the gas production in HFT are shown in Figure 1.

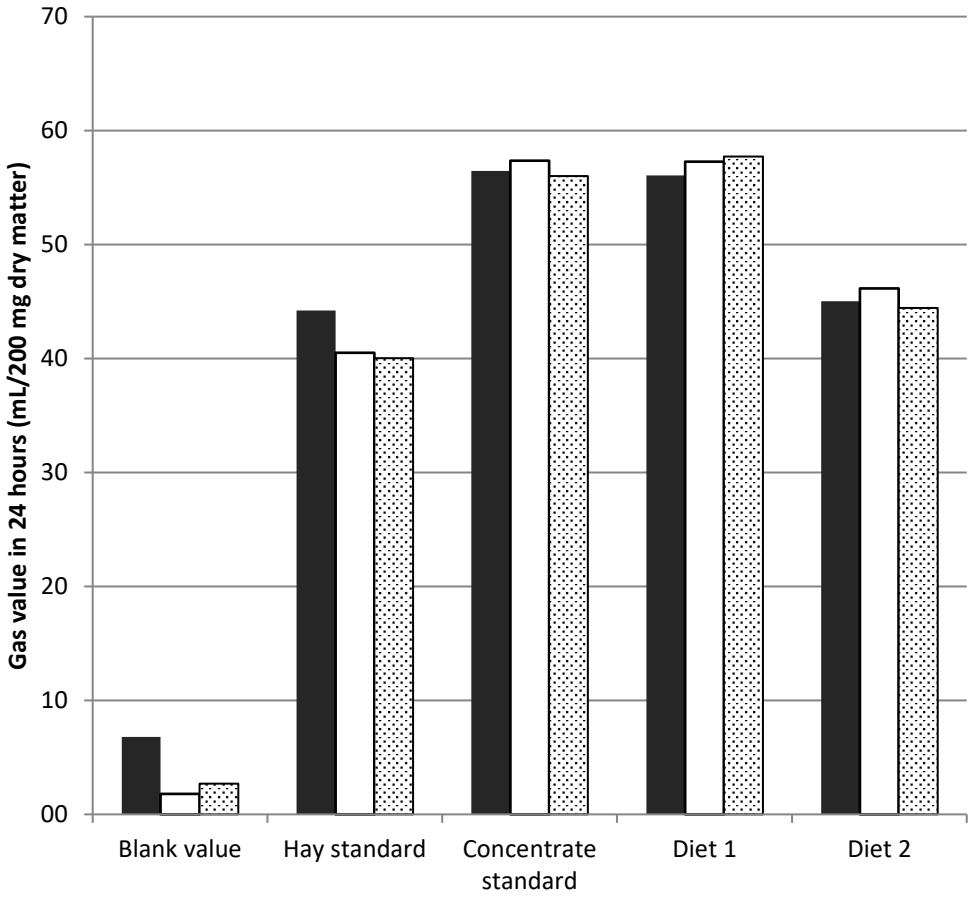

Figure 1 Gas production in 24 hours with the Hohenheim gas test using three sources of inoculum; ■ steers, □ RUSITEC fed with grass silage, and ▒ RUSITEC fed with maize silage. Next to hay and concentrate standard, a dairy compound feed (diet 1) and dried fresh lucerne (diet 2) were incubated each with three repeats.

The results of the small trial indicate the possibility of using inoculum from the RUSITEC vessel and perhaps independent of the substrate used in the RUSITEC. As well the results indicate by Barbi et al. (1993), detected that RUSITEC inoculum would appear to have the potential to replace rumen liquor as inoculum. Owen et al. (1991), applying the two-stage *in vitro* digestibility technique of Tilly und Terry (1963), also observed a strong correlation between using RUSITEC effluent and cow rumen liquor for dry matter (DM) digestibility.

If these results could be corroborated with further systematic research data, the need for ruminally fistulated animals as donors of rumen fluid could be reduced hence contributing to one of the goals of the 3-R-concept. This achievement, however, would not reduce nor replace the need for a solid inoculum for several continuous culture systems. But in this regard, strict standardization of the RUSITEC system is overdue, particularly because of its possible use as an inoculum source in ruminant nutrition research.

Recommendations for a standardised RUSITEC procedure

To facilitate a comparison of results from individual studies, we have strongly recommended standardising the technical details and methodical aspects of RUSITEC systems (Deitmers et al., 2022). The first important factor before starting RUSITEC procedures is the feeding of the donor animals. A standardised feeding of donor animals in two meals a day with a minimum between-feeding interval of 8 h should use proportions (DM basis) of 0.5 to 0.7 hay and 0.5 to 0.3 mixed concentrate with a 14-day adaptation phase. Further, rumen fluid should be collected via a vacuum pump before the morning feeding from at least two donor animals, pooled in equal parts and prepared by filtration through one layer of cheesecloth. If solid material is needed, it should be as representative as possible by using more than one sampling location, preferably in the central rumen by standardised sampler (Tafaj et al. 2001).

When the RUSITEC starts, the vessels should be filled with rumen fluid, buffer, and deionised water in a ratio of 0.5:0.2:0.1. It is recommended to use vessels with a minimum volume of 800 mL. The incubation vessel should be packed with two nylon incubation bags with a pore size of 100 µm and 13 to 16 g DM of the respective diet. At the beginning, the second bag should be filled with 80 g of rumen solids. Further it should be recommended in future to standardise the size of using incubation bags. Each bag should be incubated for 48 hours. The buffer composition proposed by McDougall (1948) is recommended. The buffer should be supplied by a peristaltic pump with a buffer flow rate of 0.5 per day (dilution rate of 2.08%/h) and the incubation bags removed from the system should be washed with 50 mL of warm buffer and squeezed moderately. The feeds incubated in the RUSITEC must be of an appropriate particle size and forages should be chopped or cut to particle lengths of no more than 3 cm while concentrates should be milled using a 1-mm sieve aperture. The introduction of a standardised approach can help to align the establishment of the RUSITEC system and make RUSITEC results more comparable both within and among laboratories.

Conclusions

This thesis has clearly pointed out, in a systematic review (Chapter 3), the lack of standardisation of RUSITEC systems and crucially presented and discussed the range of different technical approaches and procedures of the system. In addition to the technical details of several RUSITEC incubation equipments, the review clearly shows that the inoculum is also an important factor. The origin of the inoculum is influenced by the donor animals, and especially the feeding of donors and the time point of collecting ruminal contents

are crucial in this context. Therefore, not only the technical and handling procedures need revision and standardisation, but also the donor animals' feeding and the collection of inoculum need appropriate attention.

The research, in this thesis, involving a comparison of a coupled *in vivo-in situ* method with an *in vitro* RUSITEC system (Chapter 4) addresses the importance of reducing the number of animal trials. This, likewise, necessitates the development and application of novel and enhanced alternative techniques, as well as the examination of their effects on the comparability and validity of *in vivo* and *in vitro* results. The study in Chapter 4 reports on the comparability of fermentation characteristics and nutrient degradability of two common forages. The *in vitro* data seemed well comparable to the data derived from the coupled *in vivo-in situ* approach, with a high reproducibility of both methods. The RUSITEC system constituted a sufficient replacement for more laborious *in vivo* and *in situ* measurements when nutrient degradability and ranking of general fermentation characteristics of feedstuffs are the targets. However, to study diurnal fermentation patterns, the ability of the RUSITEC to accurately represent the *in vivo* situation has some limitations that need to be acknowledged.

From a methodical perspective, an effective standardisation of the RUSITEC system needs more consecutive small steps in trial designs, which advance the importance of comparison trials. A direct comparison between different types of RUSITEC systems and between RUSITEC systems with *in vivo* methods need further investigations: on the one hand about the detailed handling of the system and, on the other hand, the orientation of scientific questions as comparison to *in vivo* studies (e.g. the lack of any absorbent capacity in the RUSITEC system). Therefore, the present research may represent an incentive for future studies with the findings of this thesis being initial inputs for standardising the RUSITEC system to improve the meaningfulness of RUSITEC results for a range of purposes.

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