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The effects of a product of the solid-state fermentation of *Aspergillus niger* on feed digestibility, rumen fermentation, and rumen development in dairy cattle

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Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy

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Abstract

This thesis examines the effects of a product of the solid-state fermentation of *Aspergillus niger* (ANP) on *in vitro* digestibility of dairy rations, *in sacco* degradation of feeds and rumen fermentation in mature dairy cattle, and the growth performance (weight gain, feed intake and efficiency) and rumen development of dairy calves. A narrative review examining fungal feed additives in dairy cattle, and a systematic review examining enzymatic products in dairy calves, were also carried out. The reviews demonstrate the utility of such products to improve digestibility and growth performance variables in cattle but highlight that product efficacy can be limited by animal, dietary and environmental factors. In the first *in vitro* experiment of this project, doses of 2 and 5 mg ANP/g substrate increased total gas production from a grass silage-based ration but did not affect gas production from a maize silage-based ration. Modelled degradation kinetics of both rations were unaffected by treatment, as was volatile fatty acid production and dry matter disappearance of substrates. Four doses of ANP (0, 5, 10, 15 g/d) were trialled in fistulated dairy cattle. Treatments did not affect rumen fermentation, or the *in sacco* degradation of steam flaked barley, grass silage, or a grass silage-based total mixed ration. The effects of ANP, alone, or in combination with the mannan-rich fraction of *Saccharomyces cerevisiae* (MRF) were measured in dairy calves. Calves fed ANP alone had increased starter intake throughout the pre-weaned and weaned periods. Feed efficiency was not consistently improved by either treatment, and weight gain and faecal health were unaffected. Decreased acetate-to-propionate ratios in rumen fluid were observed in both treatment groups, but at different stages of calf development, ANP + MRF in the pre-weaned period, and ANP alone post-weaning. Both treatment groups had thicker layers of stratum basale, stratum spinosum and stratum granulosum in their rumen papillae, pre-weaning and six weeks post-weaning. This finding could indicate an increase in the absorptive and metabolic capacity of papillae, however, no coincident effects on calf performance were observed. In addition to the main calf study, the use of calf faeces as a proxy for rumen digesta in *in vitro* digestibility models was examined. There were sparse similarities between the gas production

kinetics of faecal and rumen digesta incubations, suggesting that faeces is not a suitable microbial inoculum to model digestibility in calves.

Table of Contents

Abstract 2

List of Tables 8

List of Figures 11

List of Accompanying Material 14

Acknowledgement 16

Author's Declaration 17

Definitions/Abbreviations 18

Chapter 1. Introduction 21

Chapter 2. Review: Fungal feed additives for dairy cattle, fermented fad or filamentous fortune? 30

2.1 Abstract 30

2.2 Cattle and fungi 31

2.3 Classification, production and testing of fungal feed additives 32

2.4 Anaerobic fungi 35

2.5 Obligately aerobic fungi 45

2.6 Yeasts 59

2.7 Conclusion 62

Chapter 3. The effects of a product of the solid-state fermentation of *Aspergillus niger* on the *in vitro* rumen fermentation kinetics of rations fed to dairy cattle…. 64

3.1 Abstract 64

3.2 Implications 65

3.3 Introduction 65

3.4 Materials and methods 67

3.4.1 Study design 67

3.4.2 Statistical analysis of data....70
3.5 Results....71
3.6 Discussion....79
3.7 Conclusion....81
Chapter 4. The effects of a product of the solid-state fermentation of *Aspergillus niger* on *in sacco* degradation of feeds and rumen volatile fatty acid production in dairy cattle....82
4.1 Abstract....82
4.2 Introduction....83
4.3 Materials and methods....85
4.3.1 Study design....85
4.3.2 Modelling and statistical analysis of data....88
4.4 Results....89
4.5 Discussion....100
4.6 Conclusion....103
4.7 Annex A....103
Chapter 5. Systematic review: Efficacy of exogenous enzymes for dairy calves…….....106
5.1 Abstract....106
5.2 Introduction....108
5.2.1 Rationale....108
5.2.2 Calf rearing in the dairy industry....109
5.2.3 Neonatal calves....110
5.2.4 Transitioning from pre-ruminant to ruminant....111
5.2.5 Exogenous enzyme production and classification....112
5.2.6 Justification....114
5.3 Materials and methods....115

5.4 Results 118

5.5 Discussion 132

5.5.1 *In vitro* studies 132

5.5.2 Growth and feed intake 134

5.5.3 Nutrient digestibility 143

5.5.4 Rumen characteristics and contents 145

5.5.5 Feeding economics 146

5.5.6 Behaviour 147

5.5.7 Calf health status 149

5.6 Conclusion 152

Chapter 6. Effects of an *Aspergillus niger* fermentation product and yeast mannan-rich fraction on dairy calf performance and rumen development 153

6.1 Abstract 153

6.2 Introduction 154

6.3 Materials and Methods 155

6.3.1 Treatments, animals and management 155

6.3.2 Growth, digestibility and health measurements 157

6.3.3 Rumen variables 158

6.3.4 Statistical analysis 161

6.4 Results 163

6.4.1 Intake, growth and faecal variables 163

6.4.2 Rumen variables 165

6.5 Discussion 171

6.6 Conclusion 175

Chapter 7. Comparison of calf faeces and rumen digesta as inoculum to measure *in vitro* digestibility using the gas production technique 176

7.1 Abstract 176

7.2 Introduction..... 177

7.3 Materials and methods..... 178

7.4 Results and discussion..... 180

7.5 Conclusion..... 188

Chapter 8. Summary..... 189

Bibliography..... 202

List of Tables

Table 2-1. Summary of treatments, variables, and main effects, in studies examining the use of anaerobic fungal additives in dairy cattle.....37

Table 2-2. Summary of treatments, variables, and main effects, in studies examining the use of obligately aerobic fungal additives in dairy cattle. 47

Table 3-1. Feed composition of total mixed rations used in rumen fermentation simulation to measure the effects of Synergen in cattle.....68

Table 3-2. Chemical composition of total mixed rations used in rumen fermentation simulation to measure the effects of Synergen in cattle.....68

Table 3-3. Total gas production, gas production kinetic parameters, DM disappearance, and digestion medium pH, of 1 g DM high starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle. 75

Table 3-4. Total gas production, gas production kinetic parameters, dry matter disappearance, and digestion medium pH, of 1 g DM low starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle. 76

Table 3-5. Volatile fatty acid profiles of fermentation fluid from incubating 1 g DM high starch total mixed ration with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle. 77

Table 3-6. Volatile fatty acid profiles of fermentation fluid from incubating 1 g DM low starch total mixed ration with different doses of Synergen. for 72 hours, in a rumen fermentation simulation for cattle. 78

Table 4-1. Nutritional composition of the dry cow ration, available ad libitum to four mature Jersey heifers, and the feeds suspended *in sacco*, in a 4 × 4 Latin square design trial, measuring the efficacy of four doses of Synergen on rumen fermentation kinetics. 86

Table 4-2. Exponential decay parameters estimated by the NOWAY programme (Harbron, 1994) for the degradation of dry matter (DM), organic matter (OM),

neutral detergent fibre (NDF), and crude protein (CP) components of 5 g grass silage, grass silage-based dairy TMR, and steam-flaked barley. 96

Table 4-3. Mean rumen volatile fatty acid concentrations and proportions in four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square digestibility trial. Digesta samples were collected from the ventral sac region of each rumen at seven time points during 48-h sampling periods....99

Table 4-4 Estimates of effective degradabilities of dry matter, organic matter, neutral detergent fibre, and crude protein components of 5 g grass silage, grass silage-based dairy TMR, and steam-flaked barley incubated in the rumens of fistulated Jersey cattle. 104

Table 5-1. Selection criteria for the inclusion of literature in a Systematic review of exogenous enzyme use in dairy calves. 116

Table 5-2. Information sources and search terms used to identify literature for a Systematic review of exogenous enzyme use in dairy calves. 117

Table 5-3. Summary of papers included in a Systematic review of exogenous enzyme use in dairy calves. 121

Table 6-1 Characteristics of calf rumen histology data and transformations used to meet normality assumptions where data were non-parametric. 162

Table 6-2 Feed conversion efficiency, starter concentrate intake, average daily gain and bodyweight of Holstein Friesian bull calves fed 3 g/d Aspergillus niger fermentation extract alone (ANP), or in combination with 2 g/d of the mannan rich fraction of Saccharomyces cerevisiae cell wall (MRF), compared with control calves offered no treatments (CTRL). 164

Table 6-3 Volatile fatty acid concentrations and proportions in rumen digesta collected from Holstein Friesian bull calves fed 3 g/d Aspergillus niger fermentation extract alone (ANP), or in combination with 2 g/d of the mannan-rich fraction of Saccharomyces *cerevisiae* cell wall (MRF), compared with control calves offered no treatments (CTRL). 166

Table 6-4 Rumen weights and papillae variables in Holstein Friesian bull calves fed 3 g/d Aspergillus niger fermentation extract alone (ANP), or in combination

with 2 g/d of the mannan rich fraction of Saccharomyces cerevisiae cell wall (MRF), compared with control calves offered no treatments (CTRL). 168

Table 7-1 Estimates of gas production kinetics of 0.5 g calf starter incubated for 24 h, in the ANKOMRF system with faecal and rumen inoculum obtained from Holstein Friesian calves at 27, 48 and 90 days old. 183

Table 7-2 Coefficients of determination between in vitro gas production kinetic parameters from faecal and rumen content incubations, obtained from Holstein Friesian calves at 27, 48 and 90 days old and incubated with 0.5 g calf starter for 24 h in the ANKOM^{RF} system..... 185

List of Figures

Figure 3-1. Gas production curves of 1 g DM high starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle (ANKOM, 2018).Treatments: 0NSA = 0 mg Synergen® incubated with inoculum from donor cattle not adapted to Synergen®; 0SA = 0 mg Synergen® incubated with Synergen® adapted inoculum; 0.5SA = 0.5 mg Synergen® incubated with Synergen® adapted inoculum; 2SA = 2 mg Synergen® incubated with Synergen® adapted inoculum; 5SA = 5 mg Synergen® incubated with Synergen® adapted inoculum. 73

Figure 3-2. Gas production curves of 1 g DM low starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle (ANKOM, 2018). Treatments: 0NSA = 0 mg Synergen® incubated with inoculum from donor cattle not adapted to Synergen®; 0SA = 0 mg Synergen® incubated with Synergen® adapted inoculum; 0.5SA = 0.5 mg Synergen® incubated with Synergen® adapted inoculum; 2SA = 2 mg Synergen® incubated with Synergen® adapted inoculum; 5SA = 5 mg Synergen® incubated with Synergen® adapted inoculum. 74

Figure 4-1. Degradation curves of the dry matter (A), neutral detergent fibre (B), crude protein (C), and organic matter (D) components of 5 g grass silage incubated in the rumens of four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. Plotted lines display the degradation curves calculated using the NOWAY programme (Harbron, 1994). NOWAY estimates the degradation kinetics of feed, using the exponential decay equation described by Ørskov and McDonald (1979). Individual scatter plot points display the mean degradation values of each component, quantified at seven bag withdrawal timepoints (0, 3, 6, 9, 12, 24, and 48-h). 91

Figure 4-2. Degradation curves of the dry matter (A), neutral detergent fibre (B), crude protein (C), and organic matter (D) components of 5 g grass silage-based dairy TMR incubated in the rumens of four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d) in a 4 × 4 Latin square design digestibility trial. Plotted lines display the degradation curves calculated using the NOWAY

programme (Harbron, 1994). NOWAY estimates the degradation kinetics of feed, using the exponential decay equation described by Ørskov and McDonald (1979). Individual scatter plot points display the mean degradation values of each component, quantified at seven bag withdrawal timepoints (0, 3, 6, 9, 12, 24, and 48-h). 93

Figure 4-3. Degradation curves of the dry matter (A), neutral detergent fibre (B), crude protein (C), and organic matter (D) components of 5 g steam flaked barley incubated in the rumens of four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. Plotted lines display the degradation curves calculated using the NOWAY programme (Harbron, 1994). NOWAY estimates the degradation kinetics of feed, using the exponential decay equation described by Ørskov and McDonald (1979). Individual scatter plot points display the mean degradation values of each component, quantified at seven bag withdrawal timepoints (0, 3, 6, 9, 12, 24, and 48-h). 95

Figure 4-4.Rumen pH in four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. pH of digesta was measured at seven time points during 48-h sampling periods. Error bars display SEM, and *P* values were calculated using Repeated Measures Two-way ANOVA in R.....98

Figure 5-1. PRISMA diagram displaying the number of articles assessed and included in Systematic review of exogenous enzyme use in dairy calves. 119

Figure 5-2. Variables measured, and significant effects realised in studies examining the effects of exogenous enzymes in dairy calves. Papers were identified by Systematic review following the PRISMA (2020) framework..... 120

Figure 6-1 Measurements used to quantify rumen papillae morphology in pre-weaned and weaned dairy calves. Papillae width = average W1: W5, W1 = transection point of papilla. Papillae length (L) = straight line from W1 – tip, or polyline if a straight line that passes through the papilla cannot be drawn due to bowing. Stratum corneum (SC) thickness = average SC1: SC5. Strata basale, spinosum, granulosum (SBSG) thickness = average SBSG1: SBSG5. Lines for SC and

SBSG were drawn perpendicular to the junction between SC and stratum granulosum..... 160

Figure 7-1 Gas production from 0.5 g calf starter incubated for 24-h with faecal and rumen inoculum obtained from Holstein Friesian bull calves. Solid lines show mean gas production measured at 10 min intervals using the ANKOM^{RF} GP system. Scatter points show GP from individual modules at 2 h intervals. A: Inoculum obtained from five 27 ± 5 d old calves. B: Inoculum obtained from ten 48 ± 5 d old calves. C: Inoculum obtained from ten 90 ± 5 d old calves. 182

List of Accompanying Material

Six chapters of this thesis are published papers or papers that have received final acceptance for publication and were in press at the time of submission of this thesis. The complete references, DOIs of papers and associated supplementary data, are provided here:

Chapter 2

Yerby, S., Warren, H. and Jonsson, N., 2026. Review: Fungal feed additives for dairy cattle, fermented fad or filamentous fortune? *Journal of Dairy Research,* 93, pp 335-351.

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Chapter 3

Yerby, S., Huntington, J., Warren, H. and Jonsson, N., 2025. The effects of a product of the solid-state fermentation of Aspergillus niger on the in vitro rumen fermentation kinetics of rations fed to dairy cattle. *Animal-Open Space,* 4, p.100098.

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Chapter 4

Yerby, S., Huntington, J., Warren, H. and Jonsson, N., 2025. The effects of a product of the solid-state fermentation of Aspergillus niger on *in sacco* degradation of feeds and rumen volatile fatty acid production in dairy cattle. *Journal of Dairy Research,* 92, pp. 501–509.

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Chapter 5

Yerby, S., Taylor, D., Warren, H. and Jonsson, N., 2025. Board Invited Systematic Review: Efficacy of exogenous enzymes for dairy calves. *Journal of Dairy Research*, 92, pp. 478–494.

Paper DOI: https://doi.org/10.1017/S0022029925101350

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Chapter 6

Yerby, S., Huntington, J., Jonsson, N. and Warren, H., 2026. Effects of an Aspergillus niger fermentation product and yeast mannan-rich fraction on dairy calf performance and rumen development. *Journal of Dairy Research,* 93, pp 442-449.

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Chapter 7

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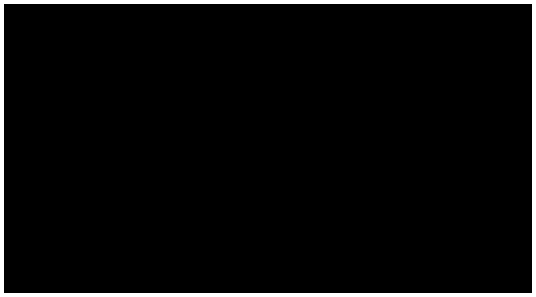
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I dedicate this thesis to my grandparents, Joan, Ted and Elaine. With sadness that you have not seen this journey, but I hope to have made you proud.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at any other university.

Name: Stefan Yerby

Signature: 

Definitions/Abbreviations

Ace:Prop	Acetate-to-propionate ratio
ADF	Acid detergent fibre
ADG	Average daily gain
ADL	Acid detergent lignin
AF	Anaerobic fungi
AIA	Acid insoluble ash
ANP	*Aspergillus niger* fermentation product
ANOVA	Analysis of variance
AOAN	*Aspergillus niger* and *Aspergillus oryzae* co-cultivation product
BCVFA	Branched chain volatile fatty acids
BW	Body weight
CF	Crude fibre
CFU	Colony forming units
CHO	Carbohydrates
CMCU	Carboxymethyl cellulase
CP	Crude protein
CPI	Crude protein intake
CTRL	Control
DFM	Direct fed microbials
DM	Dry matter
DMD	Dry matter disappearance
DMI	Dry matter intake
DNS	Dinitrosalicylic acid
ECM	Energy corrected milk
EE	Exogenous enzymes

EFE	Exogenous fibrolytic enzymes
F:C	Forage-to-concentrate ratio
FCE	Feed conversion efficiency
FCE	Feed efficiency
GC	Gas chromatography
GH	Growth hormone
GHG EI	Greenhouse gas emissions intensity
GHR	Growth hormone receptor
GI	Gastrointestinal
GP	Gas production
GPT	Glutamic pyruvic transaminase
HMGCS1	3-Hydroxy-3-Methylglutaryl-CoA Synthase 1
HPLC	High-performance liquid chromatography
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
LAB	Lactic acid bacteria
ME	Metabolizable energy
MOA	Mode of action
MOS	Mannan-oligosaccharides
MR	Milk replacer
MRF	Mannan-rich fraction of yeast cell walls
N	Nitrogen
NDF	Neutral detergent fibre
NNOB	Non-nutritive oral behaviour
OM	Organic matter
Pap	Papillae
PUN	Plasma urea nitrogen

RM	Repeated measures
SBSG	Strata basale spinosum and granulosum
SC	Stratum corneum
SD	Standard deviation
SE	Standard error
SI	Small intestine
SmF	Submerged fermentation
SSF	Solid-state fermentation
TCA	Trichloroacetic acid
TDN	Total digestible nutrients
TFU	Thallus-forming units
TMR	Total mixed ration
tVFA	Total volatile fatty acids
VFA	Volatile fatty acids
XU	Xylanase units
XYL	Xylanase

Chapter 1. Introduction

The overarching aim of the research included in this thesis was to assess the effects of a mixed fermentation product of *Aspergillus niger* (ANP) on rumen fermentation kinetics *in vitro*, *in sacco* in mature dairy cattle, and *in vivo* in transition stage dairy calves. This research aimed to increase the evidence base around the principles of ANP use in cattle by examining interactions between ANP and diet, fermentation pathways, and the histology of the developing rumen. In completing this work, we hoped to determine the stage of cattle maturity (pre-ruminant, transition stage, mature) in which ANP could be beneficial, as well as optimal dose rates and dietary conditions for ANP efficacy. The findings of this research will help to inform guidelines for the commercial use of the product investigated and contribute to the wider knowledge base surrounding ANP use in ruminants.

Aspergillus niger is a haploid filamentous fungus with a global distribution, occurring naturally as a food spoilage organism (Alkhalifah et al., 2022). The fermentative capabilities of *A. niger* have allowed it to become an important organism for biotechnology; economically vital for the food and pharmaceutical sectors due to its capacity to metabolise glucose and sucrose into citric acid, accounting for over 90 % of production worldwide (Latif et al., 2025). *Aspergillus niger* has highly efficient protein secretory mechanisms and codes for a suite of diverse enzymes, allowing it to catalyse the hydrolysis of a wide range substrates into simple sugars (Plumridge et al., 2004). This hydrolytic capacity can be exploited to release valuable molecules from biopolymers, or enzymes and metabolites produced during fermentation can be extracted from media and used for other biotechnological processes (Pel et al., 2007). The production of fungal fermentation products is discussed in detail in the next chapter, but commonly, fungi are fermented on a substrate in the presence of water (submerged-state fermentation) or in the absence of water (solid-state fermentation) (Subramaniyam and Vimala, 2012). The desired metabolites or enzymes can then be extracted from the media, or the whole fermentation

media, containing substrate, products of fermentation and organism, can be processed as a mixed fermentation product (Parmar et al., 2019).

The use of *Aspergillus* products in the ruminant feed sector is not well established compared with their use in food and pharmaceutical processing (Uwineza et al., 2024). However, there are several attributes of ANP that could be beneficial for cattle nutrition and production. As a feed ingredient, filamentous fungi are rich in essential amino acids, lipids and minerals, although they can be lacking in methionine (Karimi et al., 2021). Their penetrative hyphae, and capacity to code for many enzymes, mean that *A. niger* can increase the pre-feeding digestibility, and nutritive value, of low-quality by-products and crop residues (Ong et al., 2004), enhancing the circular bioeconomy and sustainability of ruminant production. Organic acids and residual enzyme activities present in mixed fermentation products could interact with endogenous fermentation pathways in the rumen (Kong et al., 2021), which may have advantageous outcomes for feed efficiency and productivity. Although the extensive range of substrates that *A. niger* can catalyse the hydrolysis of has made it an asset for biotechnology, this versatility also presents challenges when considering the use of ANP in cattle diets. The composition of mixed fermentation products will be dependent on the fermentation substrate, medium, and environmental conditions of fermentation (Braaksma et al., 2009). Consequently, optimal dose rates, substrate specificity, and effects on fermentation kinetics require defining for individual ANP, to inform if, and how, they can be used to enhance cattle performance. Synergen® (Alltech Inc., Kentucky, US), is a commercially available ANP, it has a crude protein (CP) content of 21 % and is produced via solid-state fermentation (SSF) of *A. niger* on wheat bran. Research presented in this thesis explores if the dietary inclusion of this ANP could be beneficial for digestibility and fermentation variables in mature dairy cattle, and performance and rumen development variables in dairy calves.

The primary objectives of this work were to:

1. Measure the effect of ANP dose on the *in vitro* fermentation kinetics of rations formulated for dairy cattle.

2. Model the effect of ANP on the degradation kinetics of feeds and feed components in the rumen.
3. Assess the impact of ANP inclusion on rumen fermentation pathways *in vitro* and *in vivo*.
4. Measure the effects of ANP on growth, feed efficiency, gut health and rumen development in dairy calves.

The secondary objectives of this work were to:

1. Consolidate and evaluate published literature examining the dietary inclusion of enzymatic products in dairy calves.
2. Assess any synergistic or additive interactions between ANP and a yeast cell wall fraction when fed to dairy calves in synchrony.
3. Evaluate whether calf faeces could be a suitable proxy for rumen digesta to model calf rumen digestibility in an *in vitro* gas production system.

The gas production technique was used to investigate the effects of ANP on *in vitro* rumen fermentation kinetics in this research. This technique allows the rate and extent of fermentation of feedstuffs to be estimated based upon the amount of gas produced when incubated with microbial inoculum and buffered medium. *In vitro* gas production has a rich history of use and adaption across institutes, spanning many decades; with early studies including McBee (1953), who measured the gas produced from the fermentation of cellulose and hemicellulose in sheep rumen fluid, to evaluate rumen microbial activity. To achieve this they incubated rumen fluid with substrate and buffers in Warburg flasks, and measured the height of water displaced in manometers to quantify the amount of gas produced during fermentation (McBee, 1953).

Since the principles of using *in vitro* gas production to estimate the fermentation of feedstuffs were established, the apparatus used to perform the technique have evolved and diversified (Rymer et al., 2005). Rather than using water displacement to measure gas production, Czerkawski and Breckenridge (1975) used the displacement of a plunger within a syringe as a method to quantify the effect of methane inhibitors on *in vitro* rumen fermentation. This technique formed the basis of the Hohenheim gas test (Menke et al., 1979), where gas

production from the incubation of rumen liquor, buffer and substrate is measured in graduated syringes at set time intervals, allowing for the estimation of the rate and extent of feed degradation.

Manual measurement of gas production, through the observation of water or syringe plunger displacement, is labour intensive and subject to human error, making automation desirable. Wilkins (1974) describes a method to measure the gas produced by microbes using a pressure transducer. In their research, gas accumulation, and consequently increased pressure, from incubations of coliform bacteria was accurately and routinely recorded (Wilkins, 1974). To estimate the rumen fermentation kinetics of feedstuffs, Theodorou et al. (1994) describes a methodology where a pressure transducer is used to record the gas pressure in the headspace of vessels containing microbial inoculum, feed substrates and culture media. Theodorou et al. (1994) observed that gas pressure and gas volume were linearly related up to a pressure of 10 psi. Consequently, they conclude that vessels should be vented and returned to ambient pressure at no more than 7 psi, arguing that pressures above this threshold could negatively affect microbial growth and substrate fermentation (Theodorou et al., 1994).

The *in vitro* gas production technique is a relatively simple method to simulate microbial fermentation through manual, semi-automated or fully automated measurement of gas production and/or gas pressure (Theodorou et al., 1994, Pell and Schofield, 1993, Menke et al., 1979). However, it does not allow for the simulation of chewing, rumen motility, feeding or the outflow and absorption of fermentation products from the rumen (with the exception of the continuous culture Rusitec system (Czerkawski and Breckenridge, 1977)). Additionally, there are several factors that can influence the estimates of degradation and fermentation across experiments, including: substrate and substrate preparation method; apparatus and experimental technique; incubation media composition; and microbial inoculum source and activity (Gierus et al., 2008, Rymer et al., 2005). In their comprehensive review, Rymer et al. (2005) identifies the greatest cause of variation in the gas production technique as source and activity of microbial inoculum. This highlights the need for consistent sampling techniques

within laboratories and encourages caution when comparing results from studies using diverse inocula.

The Theodorou et al. (1994) methodology is the basis for the ANKOM^{RF} gas production system used in this thesis. Instead of manually venting vessel headspace, the technology automatically measures headspace pressure at 10-minute intervals and opens vent valves when headspace pressure reaches at a set threshold (we used 7 psi). This technique was selected to initially investigate the effects of a range of doses of ANP on the fermentation kinetics of diets fed to dairy cattle. For dietary interventions such as ANP, *in vitro* gas production can be a valuable screening tool. This is because it allows for a large range of experimental treatments to be investigated under highly controlled conditions, potentially providing insights into the design of future *in vivo* studies; whilst avoiding much of the cost and ethical considerations required for animal trials. The ANKOM^{RF} system produces a large amount of data (432 pressure readings during a 72-hour incubation), allowing for the effects of dietary interventions on feed fermentation kinetics to be modelled and examined.

A limitation of the ANKOM^{RF} system, compared to sealed gas production techniques such as the Hohenheim gas test, is that it vents fermentation gases into the atmosphere. This prevents composition analysis of the entire gas pool produced during incubations, which may limit its use for the investigation of interventions that could affect methane production. In an experiment not included in this thesis, I attempted to collect gas from the system by attaching inert bags to the venting outlets of each vessel. However, this resulted in peculiar, and sometimes oscillating, gas production curves, suggesting a serious flaw in the methodology.

To investigate the effects of ANP on *in vivo* rumen degradation kinetics the *in sacco* artificial bag technique was used. This technique involves suspending bags containing substrates into the rumen of fistulated animals, withdrawing them sequentially, and performing proximate analysis on residues. The method was first described by Quin et al. (1938) who suspended feed samples in the rumen of fistulated sheep in cylindrical silk bags, allowing for the rate of degradation

of feedstuffs to be estimated. Subsequent researchers have used artificial bags, typically made of nylon or polyester, to perform this technique (Mehrez and Ørskov, 1977, Van Keuren and Heinemann, 1962, Kristensen et al., 1982).

The *in sacco* (or *in situ*) methodology allows for the rate and extent of feed and feed components within the rumen to be estimated. This makes it a useful tool for studying the degradation of feeds, and the effects that dietary interventions such as ANP may have on degradation kinetics through affecting the rumen environment. Suspending feeds *in sacco* exposes feeds to the rumen and its contents, however, due to confinement within a bag, feeds cannot be ruminated. The methodology produces degradation, rather than digestibility, data, as what is being measured is the rate and extent to which feed is degraded into small enough particulate to exit the bag, rather than being digested into chemical components (Ørskov and McDonald, 1979).

Mehrez and Ørskov (1977) investigated sources of variation when using the *in sacco* technique to estimate the degradation of dry matter (DM) and nitrogen (N) components of barley suspended in the rumen of fistulated sheep. They found the greatest source of variation to be between animals (DM = 6.2 %; N = 14.7 %), while smaller variation was observed between days in the same animal (DM = 4.9 %; N = 4.5 %), and between bags removed on the same day from the same animal (DM = 3.3 %; N = 6.5 %). Bag size also affects estimates of degradation. Mehrez and Ørskov (1977) observed a DM disappearance of barley incubated in 5 × 8 cm of 37.5 %, compared to 85 % disappearance in barley incubated in 17 × 9 cm bags. Ørskov and McDonald (1979) emphasize that optimum bag size is a trade off between having a suitably large surface area relative to sample size, whilst maintain a small enough bag which can be easily removed through a fistula. Notably, much of the early *in sacco* research took place in sheep, and this trade off is less pronounced when using fistulated cattle. In their recommendations for standardizing the *in sacco* methodology, Vanzant et al. (1998) suggest a bag size-to-sample size ratio of 10 mg/cm^2, bag porosity of 40 to 60 µm, and that bags be made from polyester. Additional factors affecting the estimates of degradation of feed when using this technique include: animal diet; replication; substrate preparation and the location in the rumen in which bags are suspended (Ørskov

and McDonald, 1979, Vanzant et al., 1998). Within experiments these factors should be controlled, and in publications, the methods used should be described in detail, to allow for comparison and repetition between institutes.

In their discussion of potential future applications of the artificial bag technique, beyond feed ranking, Ørskov and McDonald (1979) suggest its applicability to studying effects of changes in the rumen environment on feed degradation. Subsequently, the technique has been used to study the effects of dietary interventions such as: essential oils (Nanon et al., 2014), tannins (Hervás et al., 2003), direct fed microbials (Nocek et al., 2002) and *Aspergillus oryzae* extracts (Caton et al., 1993), on feed degradation kinetics. As such, we justify applying the technique to investigate if ANP affects the rumen environment in such a way that influences the degradation of feeds typically fed to dairy cattle.

Time series data collected from both *in vitro* gas production and *in sacco* artificial bag techniques can be used to estimate kinetic parameters of feed digestion in ruminants. To quantify the rate and extent of digestion, gas production or *in situ* feed residual data require modelling, using parameters that reflect the biological principles of the rumen (Mertens, 1993). These estimations are useful for nutritional research, as the parameters (rate and extent of digestion), represent the availability of nutrients to the animal (López et al., 1998). Data from the *in vitro* gas production technique is relatively easy to collect and experimental conditions can be highly controlled. However, data can equally be limited by experimental conditions, such as shocking microbes with aerobic conditions during microbial inoculum preparation and disparities between *in vitro* and *in vivo* conditions such as nutrient outflow and rumen motility (Mertens, 1993, Gierus et al., 2008).

Data obtained from the artificial bag technique has the advantage of representing some of the animal factors that influence digestibility, such as rumen pH fluctuations, feeding, a dynamic microbiome and the outflow of fermentation products in the environment in which the bags are suspended (Mertens, 1993, Valente et al., 2015). However, conditions within an animal are not steady state, and feeding, rumen pH and microbial dynamics can all affect digestibility kinetics. This means that estimates can be biased by time of bag

insertion relative to feeding time, and data can be further distorted by microbial attachment to feed residues and inflow and outflow of fine particulate in bags (Mertens, 1993). Models, by their very nature, do not duplicate real world conditions. Using *in vitro* and *in sacco* data to build kinetic digestibility models is useful, but, as described above, there are factors preventing *in vitro* and *in sacco* techniques from completely duplicating conditions within the rumen. As such, contextual interpretation of data derived from these models is important; effects should not be overstated, and the conditions in which they were observed defined, as well as the limitations of the methodologies used properly acknowledged.

The work described in this thesis was intended to investigate the knowledge gap surrounding ANP use in ruminant nutrition, using the *in vitro* gas production technique, the *in sacco* artificial bag technique, and an *in vivo* calf performance trial. Investigation of dietary interventions that could improve the efficiency of ruminant production are increasingly important across environmental, sustainability and economic agendas. The world's population is predicted to rise to 9 billion by 2050 (UN, 2022) and the demand for animal-based products is anticipated to double (Gouel and Guimbard, 2019), hence the need for sustainable and efficient ruminant production to meet this demand is irrefutable. An increase in animal productivity alone does not infer an increase in sustainability (or efficiency). Ruminants occupy a distinct, and valuable, niche in the global food system, for strategies to increase the output of meat and milk to be sustainable, they should not harm the environment through increased emissions intensity or biodiversity loss (Pulina et al., 2017). If diet is used to drive productivity, the inclusion of potentially human edible feeds should be minimized, and deforestation should not be used as means to increase land availability to grow ruminant feeds (Schader et al., 2015). Ruminant physiology enables them to convert feedstuffs otherwise inedible to humans, into nutrient rich, human-edible foods. Thus, by-product feedstuffs from food processing, and landscapes only capable of forage production, can be utilised by ruminants, increasing human food production and decreasing waste from other food systems (Salami et al., 2019). The rumen, the largest of the four compartments of the

ruminant forestomach, is an anaerobic fermentation chamber, populated with a diverse and dynamic microbiome often referred to as a superorganism (Krehbiel, 2014, Morgavi et al., 2013). The ruminant superorganism has a synergistic relationship with its host, by facilitating the breakdown of plant polysaccharides through substrate specific alloenzymatic digestion. This enables ruminants to derive most of their required nutrients from the products of the fermentation of plant matter, including microbial protein, volatile fatty acids, and water-soluble vitamins (Krehbiel, 2014). However, the ruminant digestive system is inefficient, with high levels of nutrients, some of which are environmental pollutants, being excreted, as well as the formation of anthropogenic greenhouse gases from microbial fermentation (Davison et al., 2020). These inefficiencies present both economic and environmental challenges. As such, the investigation of dietary interventions such as ANP, which could increase the hydrolytic capacity of the rumen through its residual enzymatic activities, is important, because of the opportunity these products could offer to enhance the efficiency of ruminant production.

Chapter 2. Review: Fungal feed additives for dairy cattle, fermented fad or filamentous fortune?

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2.1 Abstract

Fungal feed additives offer a potential avenue to enhance dairy cattle health and productivity. This narrative review examines and consolidates literature investigating the effects of products containing, or derived from, anaerobic fungi (AF), obligately aerobic filamentous fungi, and yeasts. There is a sparsity of research investigating supplemental AF in cattle, but existing literature demonstrates that AF can consistently increase the digestibility of feeds. Anaerobic fungal additives are limited by their oxygen vulnerability, although characterization of the resting stage of their lifecycle would aid in the development of products which could be used on farm. The majority of research investigating aerobic fungi in dairy cattle is based upon their fermentation products. These products often enhance digestibility variables in cattle, which sometimes benefits growth and production. Inconsistent responses, which are attributed to heterogenous product composition and rumen stability, hinders the industry utility of these additives, and further research is required to define the conditions required for efficacy. A large body of research exists examining the effects of active yeast and yeast derivatives in mature cattle and calves. In-depth examination of this research is beyond the scope of this review, but substantial evidence suggests that supplementing dairy cattle with yeast can be advantageous for gut health and development, production and stress tolerance. This review highlights the pathways through which exogenous fungi can enhance dairy production, and that further research is required to define the causes of

inconsistent responses from treatments. This will increase the value of fungal additives to the dairy sector.

Key words: aerobic fungi; anaerobic fungi; bovine, yeast, feed additive.

2.2 Cattle and fungi

Anaerobic zoosporic fungi inhabit the rumen of cattle where they engage in a symbiotic relationship with their host. First characterized by Orpin (1975), these fungi have likely inhabited, and evolved in, the gastrointestinal (GI) tracts of large herbivores for millions of years (Theodorou et al., 1996). In the rumen, fungi play a pivotal role in the degradation of recalcitrant fibrous feeds; as such, they are typically more abundant in animals fed low quality, high fibre, diets (Hartinger and Zebeli, 2021). Fungi are estimated to make up 8 to 20 % of microbial biomass (Orpin, 1981, Rezaeian et al., 2004), and provide the host ruminant with VFA, amino acids and vitamins (Hartinger and Zebeli, 2021). Although fungi have long been intertwined with sustaining cattle life, only recently has the utility of exogenous fungi, their components and their fermentation products, been realized for enhancing animal health and production in the dairy sector. Fungi can contain and produce a multitude of beneficial products including enzymes, amino acids, citric acid, polyphenols, mannan, adenosine, and sterols (Chuang et al., 2020). These compounds can have powerful antioxidant, anti-inflammatory, hydrolytic, and pro- and prebiotic effects in cattle, leading to enhanced immune function, rumen fermentation, growth, GI tract development, fertility and lactational performance (Beev et al., 2007, Varada and Kumar, 2024).

Approximately 150,000 species of fungi have been classified to date (Bhunjun et al., 2022); most of these species are probably not beneficial in the diets of cattle, and some are pathogenic and/or produce mycotoxins which can have negative ramifications for cattle health and productivity (Gallo et al., 2015). Species that have been used in, or to produce, feed additives for cattle include anaerobic fungi (AF): *Piromyces* spp., *Orpinomyces* spp., *Anaeromyces* spp., *Neocallimastix* spp.; obligately aerobic fungi: *Trichoderma* spp., *Aspergillus niger, Aspergillus oryzae*; and yeast: *Saccharomyces cerevisiae* (Varada and

Kumar, 2024, Król et al., 2023). The aim of this review is to consolidate literature investigating the utility of fungal feed additives in cattle, examine their benefits and limitations, and highlight areas of research which require further exploration.

2.3 Classification, production and testing of fungal feed additives

Fungal feed additives can be broadly split into mixed fermentation products, direct fed microbials (DFM) or probiotics, and purified fermentation products or cell components. Mixed fermentation products, sometimes referred to as cultures, can contain some or all of: viable or non-viable cells, cultivation media, and products of fermentation, including metabolites and enzymes (Krishna, 2005). The terms DFM and probiotic are often used interchangeably to describe additives with a live component, however, they have slightly different definitions. Direct fed microbials are defined as additives that only contain live or naturally occurring microbes (FDA, 1995), whereas probiotics are defined as live microorganisms that when administered in adequate amounts, confer a health benefit upon the host (FAO/WHO, 2001), so may contain metabolites, enzymes, and live organisms. Purified fermentation products are specific compounds or molecules that have been produced by fungal fermentation and then extracted from the fermentation media. In the context of this review, the most relevant fermentation products are enzymes, the profile of which is dependent on fungal species, fungal strain, genetic modification, cultivation method and cultivation substrate (Krishna, 2005). The use of fungal cell components in cattle nutrition is predominantly focused on yeast. The intracellular soluble component of yeast cells is a valuable source of nucleotides, vitamins, and lipids, and the insoluble cell walls are rich in mannan-oligosaccharides (MOS) and ß-Glucans (Shurson, 2018).

The production of all fungal feed additives requires fermentation. Commercial techniques are solid-state fermentation (SSF) or submerged fermentation (SmF); both involve cultivating fungi on a substrate in the presence (SmF) or absence (SSF) of free-flowing liquid. Solid-state fermentation tends to have higher yields,

cost less and have a lower risk of contamination than SmF (Jonathan et al., 2021). However, when the entire culture media is harvested from SSF to produce mixed fermentation products, there can be lack of homogeneity throughout the product, caused by difficulties agitating a solid fermentation bed (Krishna, 2005). This can be problematic when the recommended feeding doses of mixed fermentation products are low, potentially leading to heterogenous distribution of the active components and lack of efficacy in the final feedstuff. Conversely, SmF typically occurs in stirred-tank reactors, which makes achieving physical and chemical homogeneity considerably easier (Jonathan et al., 2021). The purpose of fungal fermentation can be solely to produce feed additives for livestock, or additives can be obtained from by-products of other fermentative processes, such as brewing (Shurson, 2018). To obtain specific fungal enzymes, metabolites or cell components, purification steps are required. For enzymes, this involves filtration, solvent extraction, precipitation, and chromatography (Jonathan et al., 2021). Cell components can be obtained using ultrafiltration, subcellular fractionation, enzymatic digestion, Concanavalin-A chromatography and sodium dodecyl sulphate extraction (Utama et al., 2023). Most fungal products are dried and stored anaerobically for stability. The technique for drying products with an active component is particularly important as moist heat can disrupt fungal cells; commonly used methods for drying DFM and probiotics include rotolouver, fluid-bed, and tunnel drying (Shurson, 2018).

In vitro and *in vivo* techniques are used to test the effects of fungal feed additives. When the primary mode of action (MOA) of a product is enzymatic, its activity will usually be assessed by quantifying the rate of accumulation of the product of the reaction that the enzyme catalyses when combined with a standard substrate. However, the conditions under which these assays are performed are often not representative of the conditions found in the digestive tract of cattle, where pH, temperature and inhibitory co-factors can all affect enzyme efficacy (Meale et al., 2014). The *in vitro* gas production (GP) technique overcomes some of these challenges, where rumen fermentation kinetics are modelled by measuring GP, when substrates are incubated with microbial inoculum derived from rumen digesta or fluid (Theodorou et al., 1994). The GP

technique is a high throughput method that can be used to test the effects of feed additives on digestibility and gas composition, providing a better insight into the effects that enzymatic products could have *in vivo*, compared to gas chromatography, high-performance liquid chromatography, or dinitrosalicylic acid assays. *In vitro* GP does not mimic rumen motility or mastication, and the microbial diversity present in inoculum may not be typical of the populations in the rumen, especially if rumen fluid that excludes protozoa and most fungi is used, limiting the conclusions that should be drawn from GP experimentation (Yáñez-Ruiz et al., 2016). When the MOA of fungal products is pre- or probiotic, digestive tract simulations are equally valuable. These assays focus on screening product viability and actions under conditions found in the stomach and small intestine; for cattle, these assays are less developed than rumen simulations and present a valuable area of further refinement. Intestinal conditions to measure pH and bile stability can be mimicked using incubations of potassium dihydrogen phosphate, bovine bile, calcium chloride and pancreatin (Vinyard et al., 2021). Haemolytic and immunomodulation activities can be measured using cell and co-cultures to examine the effects of additives on immune cells, epithelial structure and integrity, and bacteria (Papadimitriou et al., 2015). When fungal additives contain viable cells, activity is usually expressed as colony- (CFU) or thallus-forming units (TFU), per unit weight of product (Król et al., 2023). Colony-forming unit is a measure of the number of cells able to reproduce on a media to form colonies rather than a measure of cells viability *per se*. Interpretation can be confounded by the fact that colonies can be formed from one cell or from multiple cells that have aggregated, and the incidence of aggregation is increased following heat treatment, for example pelleting feed (Kwolek-Mirek and Zadrag-Tecza, 2014).

In vivo experimentation is the gold standard for testing fungal feed additives in cattle. Using animals in research is expensive, time consuming, and requires ethical consideration. Hence, pre-screening products using *in vitro* techniques to determine potential utility and optimal inclusion rates is important economically and ethically, before proceeding to animal studies. *In vivo* experiments can measure the effects of treatments on primary response variables such as growth,

health and lactation, with these variables likely to influence the industry uptake of additives. However, characterizing the biological mechanisms that lead to beneficial health and production responses is critical for product development and refinement. When improved rumen fermentation is the desired outcome from supplementation, ruminally cannulated cattle can be used to investigate the effects of additives on feed and feed component degradation using *in sacco* techniques (Ørskov et al., 1980). Rumen access provides the cleanest and most accurate means to determine effects on rumen microbial community composition and diversity, which is useful for products with digestibility and health MOAs. Measuring the effects of products with purported health benefits can be done non-invasively using indicators such as faecal score, behaviour, and biological markers in milk (Džermeikaitė et al., 2023). However, haematological, histological and blood biochemical variables can provide considerably more information on the effects of treatment on immunity and digestive tract health and development. Studies that can measure the effects of treatments at cellular, tissue and whole-body system levels undoubtedly provide the most comprehensive insight into the efficacy of fungal feed additives.

2.4 Anaerobic fungi

Anaerobic fungi play a pivotal role in degrading lignocellulosic compounds in the rumen and are efficient fibre degrading microbes (Hess et al., 2020). This physiological group of fungi have flagellated zoospores which reach and colonize deep inside fibrous material; making regions which are usually unavailable to fibrolytic bacteria, accessible through the physical splitting of plant tissue (Bhagat et al., 2023, Du et al., 2024). This penetrative capability is compounded by the remarkable suite of enzymes that AF encode for, including polysaccharidases, glycosidases, esterases, and pectinolytic lyases, which are employed to rapidly hydrolyse fibre components entering the rumen (Bhagat et al., 2023). Given that cell wall digestibility in the rumen is limited to 60 % under ideal conditions (Van Soest, 1994), supplementing cattle with AF that have efficient cell wall degrading capabilities, could be a useful strategy to enhance feed efficiency. However, it is AF’s vulnerability to oxygen that limits the

accessibility of supplemental AF as feed additives on farm. A summary of the research examining anaerobic fungal additives, discussed in this review, is shown in Table 2-1.

Table 2-1. Summary of treatments, variables, and main effects, in studies examining the use of anaerobic fungal additives in dairy cattle.

Abbreviated title	Treatments	Experimental conditions	Variables measured	Main effects	Reference
Fibrolytic potential of anaerobic fungi on *in vitro* fermentation of wheat straw and methane emissions	*Piromyces* isolates (× 10)	*In vitro* incubations with wheat straw	Apparent digestibility, true digestibility, NDF digestibility, methane, fungal count, cellulase and xylanase activities	Only observed from cattle isolates: ↑ digestibility ↑ enzyme activities Observed from cattle isolates and one Nilgai isolate: ↑ fungal biomass	Paul et al. (2010)
Potential of anaerobic rumen fungi to degrade lignified feedstuffs	*Orpinomyces joyonii* zoospores	*In vitro* incubations with wheat straw, paddy straw, and straw based TMR	DMD, ADF digestibility, NDF digestibility, enzyme activity	↑ digestibility of DM, NDF, ADF ↑ esterase activity	Kumar et al. (2012)
Isolation, characterization and fibre degradation potential of anaerobic rumen fungi from cattle	*Orpinomyces* (× 7) and *Anaeromyces* (× 13) isolates	*In vitro* incubations with wheat straw NDF	DMD, GP, partition factor, microbial biomass, VFA	↑ DMD ↑ GP ↑ microbial biomass ↑ acetate	Sirohi et al. (2013)

Effects of anaerobic fungu on *in vitro* fermentation of wheat straw	*Oprinomyces* sp. YF3	*In vitro* incubations with wheat straw NDF	VFA, ammonia-N, pH, enzyme activities, microbiome	↑ ammonia-N ↓ butyrate, propionate ↑ acetate, acetate: propionate, valerate ↑ carboxymethyl cellulase, avicelase activities ↑ abundance of Bacteroidota, Synergistota, Desulfobacterota, Actinobacteria, Fusobacteriota ↓ abundance of Fibrobacterota, Proteobacteria	Du et al. (2024)
Influence of an anaerobic fungal culture administration on growth rate, ruminal fermentation and nutrient digestion in calves	*Orpinomyces* C-14	*In vivo* (juvenile Holstein × Tharparkar, 10 months old)	DMI, weight gain, feed efficiency, TDN, digestibility of DM, OM, CP, ether extract, CF, nitrogen free extract, NDF, ADF, cellulose	↑ total weight gain, ADG ↑ digestibility of all nutrients except for ether extract ↓ rumen pH ↑ nitrogen (total N, TCA precipitable N) ↓ ammonia-N ↑ TDN	Dey et al. (2004)

Effect of administration of rumen fungi on production performance of lactating buffaloes	*Orpinomyces* sp. C-14 and *Piromyces* sp. WNG-12	*In vivo* (mature buffalo)	Milk yield, milk composition, DMI, feed efficiency, rumen pH, tVFA, zoospores, nitrogen	↑ tVFA ↑ nitrogen (total N, TCA precipitable N) ↓ ammonia-N ↑ zoospore/ml rumen fluid	Saxena et al. (2010)
Effect of feeding isolates of anaerobic fungus on growth rate and fibre digestion in buffalo calves	*Neocallimastix* sp. CF-17	*In vivo* (buffalo calves, age undeclared)	Body weight, ADG, straw intake, dietary TDN, dietary CP, digestibility of DM, OM, CP, ether extract, NDF, ADF, enzyme activity, microbial abundance, tVFA, nitrogen	↑ BW in first 4-wks ↑ ADG ↑ OM, DM, NDF, ADF digestibility ↑ TDN ↑ tVFA ↑ fungi, bacteria ↑ carboxymethyl cellulase, xylanase activities ↑ tVFA ↑ TCA precipitable N	Paul et al. (2011)
Effect of feeding fungal zoospores in TMR on	*Orpinomyces joyonii* zoospores	*In vivo* (mature buffalo)	Rumen pH, tVFA, nitrogen, zoospores,	↑ tVFA	Sehgal et al. (2012)

nutrient utilization and rumen fermentation parameters			intake. digestibility of DM, OM, CP, ether extract, CF, nitrogen free extract, NDF, ADF, cellulose	↑ nitrogen (total N, TCA precipitable N) ↓ ammonia-N ↑ zoospore/ml rumen fluid ↑ digestibility of all nutrients except for ether extract	
Growth performance of buffalo calves fed wheat straw incorporated with anaerobic fungal zoospores	*Neocallimastix* sp. GR-1	*In vivo* (juvenile buffalo, 5-7 months old)	BW, weight gain, DMI, feed efficiency, TDN, tVFA, nitrogen, zoospores, bacteria, protozoa, digestibility of DM, OM, CP, ether extract, CF, nitrogen free extract, NDF, ADF, cellulose	↑ BW, total weight gain, ADG ↑ feed efficiency ↑ digestibility of all nutrients except for ether extract ↑ TDN ↑ tVFA ↑ zoospores, bacteria, protozoa ↑ nitrogen (total N, TCA precipitable N) ↓ ammonia-N	Kumar et al. (2015)

Abbreviations: NDF = neutral detergent fibre; DM = dry matter; DMD = dry matter disappearance; GP = gas productions; VFA = volatile fatty acids; N = nitrogen; ADF = acid detergent fibre; TMR = total mixed ration; tVFA = total volatile fatty acids; DMI = dry matter intake; OM = organic matter; CP = crude protein; CF = crude fibre; BW = body weight; TCA = trichloroacetic acid; ADG = average daily gain; TDN = total digestible nutrients.

Paul et al. (2010) isolated and cultured ten *Piromyces* spp. from Nilgai (*Boselaphus tragocamelus*) and wild cattle (no breed specified) and quantified their effect on *in vitro* rumen fermentation of wheat straw, when inoculated with rumen fluid from buffalo. All five isolates obtained from wild cattle increased the digestibility of wheat straw and increased fungal biomass in incubations. However, none of the *Piromyces* isolates obtained from Nilgai increased the true digestibility of wheat straw, and only one isolate increased fungal biomass. This highlights substrate specificity differences between fungi of the same genus, isolated from cattle and Nilgai, with those cultured from cattle more useful at hydrolysing the fibre present in wheat straw. Dietary differences between Nilgai and cattle likely explain this, with Nilgai favouring forbs and shrubs, and cattle preferentially consuming forages (Sheffield, 1983). The species of *Piromyces* present in the rumens of cattle should be effective at hydrolysing the fibres their host preferentially consumes, including the wheat straw fibres investigated by Paul et al. (2010). Alternatively, limited effects from Nilgai isolated *Piromyces* could indicate that they were not equipped to survive in the microbial community present in the buffalo rumen fluid.

Sirohi et al. (2013) isolated *Orpinomyces* spp. and *Anaeromyces* spp. from rumen fluid collected from Karan × Friesian cattle and quantified the effect of incubating their cultures with NDF from wheat straw on *in vitro* fermentation. Acetate production was increased by the addition of all fungal cultures, as was the digestibility of NDF and total microbial biomass production. Incubations containing *Orpinomyces* spp. yielded increased NDF digestibility compared with incubations containing *Anaeromyces* spp., suggesting the former is more useful for enhancing fibre degradation than the latter. In accordance with this, when Du et al. (2024) introduced *Orpinomyces* sp. YF3, cultured from the rumens of Qinghai yaks, into rumen fluid from dairy goats, an increase in acetate, acetate: propionate ratio, and total volatile fatty acid (tVFA) production from the *in vitro* fermentation of wheat straw was measured. This was likely associated with the enhancement of exoglucanase and acetyl esterase activities and an increase in *Ruminococcus* and *Pyramidobacter* abundance from *Orpinomyces* addition,

highlighting a synergistic relationship between AF and fibre degrading bacteria. Although it is commercially impractical to harvest AF from one ruminant and transfer to another, this study does indicate the highly efficient fibre degrading capabilities of *Orpinomyces* sp. YF3 and its ability to propagate favourable microbial dynamics in the rumen. Kumar et al. (2012) inoculated rumen fluid with broth containing lab-cultured *Orpinomyces joyonii* zoospores, to quantity the effect of AF addition on the *in vitro* fermentation kinetics of wheat straw, paddy straw, and a TMR consisting of both straws and 40 % concentrate. AF addition increased the digestibility of DM, ADF and NDF in all substrates, and AF incubations had greater esterase activities than controls. Esterases are key enzymes involved in the hydrolysis of ester linkages between lignin and hemicellulose, thus the increase in their activities from AF supplementation likely caused the increased digestibility of substrates. The capacity of AF to enhance the digestibility of fibre has been presented by all *in vitro* studies discussed. However, maintaining anaerobic conditions to maintain AF viability in a lab is less challenging than on farm. *In vitro* experimentation is undoubtedly valuable, but for supplemental AF to have industry utility, applications must be translatable to on farm practicality and more *in vivo* studies are required to validate effects. Although AF cannot grow in the presence of oxygen, some species can tolerate aerobic environments for several hours (Trinci et al., 1988). Thus, in conditions where feed or animals can be regularly inoculated with fresh isolates that will be ingested into a favourable environment for fungal survival, their effects on performance and digestibility could be harnessed. However, most farmers would lack the time and infrastructure to culture AF and regularly inoculate cattle or feeds.

When Saxena et al. (2010) dosed buffalo with 250 ml/d broth containing *Piromyces* sp. WNG-12 or *Orpinomyces* sp. C-14 isolates, beneficial lactation responses were measured. Increases in milk yield and milk solids were realized from treatment, although the authors declared significance at $p < 0.1$ and do not state specific p values, so effects might be tendencies and not meet the more robust significance level of $p < 0.05$. Rumen fermentation variables from treatments were significantly ($p < 0.05$) enhanced, with increased rumen VFA,

total N, trichloroacetic acid (TCA) precipitable N, decreased ammonia N and zoospore count in rumen fluid. In lactating buffalo, Sehgal et al. (2012) measured enhanced rumen fermentation performance from inoculating *Orpinomyces joyonii* zoospores at 10.7 × 10^6 CFU/ml/kg feed into a wheat straw, paddy straw, concentrate, and green fodder TMR, daily. Over an experimental period of 105 d, using five buffalo per treatment, the digestibility of DM, OM, NDF, ADF and CP was increased from treatment, as was tVFA, total N, ammonia N, and zoospore count in rumen fluid. Increased energy availability from enhanced nutrient digestibilities would suggest that *O. joyonii* supplementation could improve production responses. However, feed intake and lactational performance of buffalo were unaffected, indicating that digestibility of feeds was not increased sufficiently to cause a detectable production response, or perhaps that the study was statistically underpowered. The literature examining AF supplementation in lactating cattle is sparse, but further exploration of AF effects on lactational biology is worthwhile and could improve the performance of cattle fed high levels of recalcitrant fibre. Notably, cattle on low quality fibre diets are often situated in lower income systems, where the use of any fungal additive could be limited by socio-economic factors (Balehegn et al., 2020).

Several studies have examined the effect of AF in young cattle. Because fungi are some of the first microbes to inhabit the rumen (Li et al., 2023), exogenous AF could propagate rumen microbial colonization and enhance digestive function. However, none of the studies identified by this review examined AF supplementation in pre-ruminant calves, highlighting an area for future research to examine if supplemental AF could enhance rumen development through improved solid feed degradation. Kumar et al. (2015) found beneficial effects on growth and digestibility in young buffalo aged 5 – 7 months, when incorporating *Neocallimastix* sp. GR-1 zoospores into wheat straw-based diets. Lab cultured zoospores were inoculated into straw blocks at 10^6 zoospores/kg feed over a 172-d experimental period. Animals fed fungal treated straw had improved growth rates (16 %) and feed efficiencies (28.7 %), compared to controls. Enhanced animal performance was likely caused by the increase in OM, DM, NDF, ADF, CP, crude fibre (CF), and cellulose digestibilities of wheat straw from

zoospore treatment. Dey et al. (2004) measured the effect of supplemental *Orpinomyces* C-14 in twelve 10-month-old Tharparkar × Holstein Friesian cattle on a 50:50 wheat straw: concentrate diet. Animals were orally drenched once per week with 160 ml of fungal culture containing 10^6 CFU/ml. Similarly to the observations of Kumar et al. (2015), Dey et al. (2004) reported that calves drenched with AF had greater weight gain (15.7 %) than control calves, and increased digestibilities of DM, CP, N free extracts, CF, NDF and ADF. Rumen pH and ammonia N were lower in fungal treated cattle, and total N and TCA precipitable N were increased. Physical disruption of plant tissues by *Orpinomyces* hyphae, likely caused an increase in cellulose surface area available for degradation, leading to the increase in ADG observed in this study. Notably, weekly drenching was sufficient to stimulate this effect, suggesting that supplementation altered the dynamics of the rumen microbiome for longer than the life cycle of the *Orpinomyces* C-14 administered, which would typically be 24 – 36 h (Lowe et al., 1987). The rumen microbiome is normally extremely resistant to the introduction of exogenous microbes (Weimer, 2015), but perhaps because AF are already present in the rumen, it may be easier to alter their proportions compared with the introduction of microbes which do not normally reside there. Investigations into how long AF population levels remain functionally elevated post supplementation would inform the intervals that could be employed in dosing strategies; the utility of exogenous AF in cattle would be considerably greater if dosing did not need to occur daily to realize beneficial effects. Paul et al. (2011) found that dosing buffalo calves (weight 148 ± 12 kg, age undeclared) every eight days with *Neocallimastix* sp. CF-17 zoospores and rhizomycelia, was optimal to enhance digestibility and growth rates. The first experiment in this study examined two dosing techniques over a four-week experimental period, oral dosing of 200 ml liquid AF culture containing 10^6 TFU/ml, and the same dose of AF encapsulated in an oxygen resistant biopolymer (composition undeclared). Only calves administered with encapsulated *Neocallimastix* had greater ADG than control calves. Both liquid and encapsulated treatments led to improved OM digestibility, but only encapsulated AF caused an improvement in NDF digestibility, and only liquid AF treatment calves had increased DM and ADF digestibilities. Notably, both groups

of treatment calves consumed increased TDN. When different dosing frequencies of encapsulated AF were investigated in a further experiment, there was no difference between growth rates in calves dosed every four days and calves dosed every eight days, with both groups having increased growth (20 %) compared to controls. This indicates that a dosing frequency of at least eight days can improve calf performance, and encapsulation enhanced calf growth compared with oral dosing of liquid culture. The biopolymer used by Paul et al. (2011) only afforded fungi protection from oxygen for 12 h, which would still make on farm storage challenging. Research to develop a more resistant polymer to protect AF from oxygen would increase the accessibility of AF to farmers.

Limited research examining the effects of supplemental AF in cattle has realized consistent responses in improved nutrient digestibility, which are often paired with improvements in animal performance. The fibre degrading capabilities of AF make them an appealing tool for ruminant producers and nutritionists, only hindered by challenges in handling and storage which arise from the anaerobic conditions required for AF viability. An area of research which could help overcome these challenges is the resting stage of AF's life cycle. This stage is poorly understood but is believed to allow fungi to survive for prolonged periods in aerobic conditions, and has been proposed as the reason why AF can be cultured from faeces that has been exposed to oxygen for long periods of time (Hess et al., 2020). Comprehension and characterization of the resting stage of AF could significantly expedite the development of AF additives for cattle and, as such, requires investigation.

2.5 Obligately aerobic fungi

The rumen is considered an anaerobic environment. However, small amounts of oxygen enter the rumen during feeding and regurgitation, and this is likely why obligate aerobes including *Aspergillus* and *Trichoderma* spp. can be isolated from rumen fluid (Almeida et al., 2012). Although the relative abundance of obligately aerobic fungi is small compared to AF and other microbes in the rumen, a diversity of species can persist (Wang et al., 2024). This is demonstrated by Almeida et al. (2012), who isolated 49 species from the genera:

Aspergillus, Gliocladium, Paecilomyces, Rhizophus and *Scedosporium,* from the rumen fluid of dairy cattle fed tropical forages. Outside of the rumen, obligate aerobes are predominantly used for the fermentation products that they can produce. These filamentous fungi are often characterized as 'enzyme factories' and have been used in human food fermentation for many years (Abdullahi et al., 2021). In cattle nutrition, the main focus of obligately aerobic fungi is in biotransformation to produce enzyme rich feed materials; where fungi can be cultivated on low value substrates to produce high value active compounds that can act as digestibility enhancers (Thomas et al., 2013). A summary of the research examining obligately aerobic fungal additives, discussed in this review, is shown in Table 2-2.

Table 2-2. Summary of treatments, variables, and main effects, in studies examining the use of obligately aerobic fungal additives in dairy cattle.

Abbreviated title	Treatment	Experimental condition	Variables measured	Main effects	Reference
Influence of supplemental endoglucanase or xylanase on volatile fatty acid production from ruminant feed by ruminal *in vitro* cultures	Fermentation extract of *Aspergillus niger* alone or in combination with *Trichoderma viride*	*In vitro* incubations with fescue hay, dry rolled corn, alfalfa pellets, oat by-product and a fescue hay-based TMR	VFA	*A. niger* + fescue hay, alfalfa: ↑ tVFA, acetate *A. niger* + TMR: ↑ tVFA, acetate, propionate, butyrate, isobutyrate, valerate *T. viride* + TMR ↓ acetate: propionate *T. viride* + TMR, corn, fescue hay, alfalfa: ↑ tVFA	Tricarico and Dawson (2005)
Effect of *Aspergillus oryzae* and *Aspergillus niger* co-cultivation extract on *in vitro* fermentation	Fermentation extract of *Aspergillus oryzae* and *Aspergillus niger*	*In vitro* incubations with corn silage-based TMR, alfalfa hay, oat hay and corn silage	pH, VFA, gas production, digestibility of DM, ADF, NDF, CP, microbial diversity	TMR, corn silage, alfalfa: ↑ GP ↑ DM, NDF digestibility (all substrates) TMR, corn silage, oat hay: ↑ ADF digestibility	Kong et al. (2021)

				Corn silage, oat hay, alfalfa: ↑ CP digestibility ↑ pH, acetate, acetate: propionate TMR, alfalfa, oat hay: ↑ *Prevotella* ↓ *Ruminococcus*	
Trichoderma xylanases, production, characterization and application as additives in the digestibility of forage for cattle	Fermentation extracts of *Trichoderma koningii* and *Trichoderma pseudokonigii*	*In vitro* incubations with corn silage, sugarcane silage, Tifton 85 hay, Mombaça (*Panicum maximum*), and Marandu (*Brachiaria brizantha*)	Digestibility of DM, NDF, cellulose, hemicellulose	Mombaça incubations only: ↑ DM, NDF digestibility	Sanguine et al. (2022)
Effects of exogenous fibrolytic enzymes on rumen degradation characteristics and degradability of faba bean silage in dairy cows	Fermentation extract of *Trichoderma reesei*	*In vitro* and *in sacco* incubations with Faba bean silage	Digestibility of DM and NDF	*In vitro* and *in sacco*: ↑ DM, NDF digestibility	Yang et al. (2022)

Improving the nutritional value of sesame seed coats using exogenous fibrolytic enzymes	Fermentation extract of *Trichoderma longibrachiatum*	*In vitro* incubations with sesame seed coats	GP kinetics, pH, ammonia-N, VFA, DMD, microbial protein, enzyme activity, protozoa	↑ total GP, rate of GP ↓ lag time ↑ VFA. DMD, microbial protein ↓ ammonia-N ↑xylanase, endoglucanase, exoglycanase activities ↑ protozoa	Abid et al. (2023)
Effect of yeast culture and *Aspergillus oryzae* fermentation extract on ruminal characteristics and nutrient digestibility	Fermentation extract of *Aspergillus oryzae*	*In vivo* (Holstein cows)	VFA, pH, digestibility of DM, CP, ADF, hemicellulose, microbial diversity	↑ DM, CP, hemicellulose digestibility ↑cellulolytic bacteria	Wiedmeier et al. (1987)
Effect of graded amounts of *Aspergillus oryzae* fermentation extract on ruminal characteristics and nutrient digestibility in cattle	Fermentation extract of *Aspergillus oryzae*	*In vivo* (Holstein cows)	VFA, digestibility of NDF, ADF, CP, pH, rumen microbial diversity	↓ rumen pH ↓ proteolytic bacteria	Oellermann et al. (1990)

Effect on a crude fermentation extract derived from *Trichoderma* on the performance of early lactation primiparous cows	Fermentation extract of *Trichoderma*	*In vivo* (lactating cattle, breed undisclosed)	Milk yield and composition, TMR digestibility, TMR ME, fertility	↑ milk yield ↑ digestibility, ME ↑ fertility	Walker and Povey (2016)
Effects of feeding Aspergillus oryzae fermentation product to Holstein cows	Fermentation extract of *Aspergillus oryzae*	*In vivo* ×2 experiments (Holstein cows)	Milk yield and composition, BW, DMI, plasma glucose and non-esterified fatty acids	Exp.1 ↑ milk yield Exp.2 ↓ milk yield ↑ milk fat	Sucu et al. (2018)
Improving the performance of dairy cattle with a xylanase-rich exogenous enzyme preparation	Fermentation extracts of *Trichoderma reesei*	*In vivo* (Holstein cows)	Milk yield and composition, intake, feed efficiency, DMD, pH, ammonia-N, VFA	↑ intake	Romero et al. (2016)

Supplementing dairy calves with *Aspergillus* fermentation products	Fermentation extracts of *Aspergillus niger* and *Aspergillus oryzae*	*In vivo* (Jersey and Holstein calves)	BW, weight gain, body measurements, health	No effects	Vaz-Ramírez et al. (2021)
Effect of fibrolytic enzymes on intake, nutrient utilization and economics of feeding in crossbred calves	Fermentation extracts of *Aspergillus niger*	*In vivo* (crossbred Jersey heifers)	Feed intake, FCE, BW, digestibility of DM, OM, NDF, ADF, hemicellulose, cellulose	↑ intake, FCE ↑ digestibility (all nutrients)	Anil et al. (2022)
Response of milk performance, rumen and hindgut microbiome to *Aspergillus oryzae* fermentation extracts in dairy cows	Fermentation extract of *Aspergillus oryzae*	*In vivo* (Holstein cows)	Milk yield and composition, VFA, rumen and faecal microbial diversity	↑ milk yield, protein, lactose ↑ faecal *Succinivibrio* spp.	Zhang et al. (2022)
A meta-analysis of the impact of an *Aspergillus oryzae* fermentation product on ruminal fermentation and dairy cow performance	Fermentation extract of *Aspergillus oryzae*	Meta-analysis	Milk yield and composition, feed intake, FCE, rumen fermentation	↑ milk yield, ECM ↓ milk solids ↑ DMI ↑ rumen pH, acetate: propionate	Cantet et al. (2025)

Abbreviations: DM = dry matter; NDF = neutral detergent fibre; DMD = dry matter disappearance; GP = gas productions; VFA = volatile fatty acids; N = nitrogen; ME = metabolizable energy; TMR = total mixed ration; tVFA = total volatile fatty acids; ADF = acid detergent fibre; DMI = dry matter intake; OM = organic matter; CP = crude protein; CF = crude fibre; BW = body weight; FCE = feed conversion efficiency; ECM = energy corrected milk

Trichoderma spp. are soil-borne fungi with global distribution; they synthesize a range of fibrolytic enzymes and antimicrobial compounds (Król et al., 2023). Sanguine et al. (2022) quantified the effects of xylanases produced by the SSF of *Trichoderma koningii* and *Trichoderma pseudokonigii* on the *in vitro* rumen digestibility of a range of forages. The authors only declare results from incubations using Mombaça (*Panicum maximum*), where xylanases from both *Trichoderma* spp. increased DM and NDF digestibility, suggesting that there was possibly no effect on the other feeds examined. The xylanases were found to be stable at a pH range of 3 – 9.5, and were thermostable for 1 h at 45 – 50 °C. The pH stability of exogenous enzymes (EE) can limit their efficacy in the rumen (Beauchemin et al., 2004). However, the range of stability in these *Trichoderma* EE is far greater than pH fluctuations that would occur in a healthy rumen, suggesting that stability would not limit their use *in vivo*, but limited effects on the digestibility of feeds other than Mombaça, might. When incubating 0.2 g DM sesame seed coats with *Trichoderma longibrachiatum* extract containing xylanase, endoglucanase and exoglucanase, Abid et al. (2023) measured improvements in *in vitro* rumen fermentation variables. A dose of 4 µl/g DM substrate decreased the lag time for the start of fermentation, and increased the rate of GP, DM disappearance (DMD), VFA production, hydrolysis of NDF, and rumen fibrolytic enzyme activity. Whereas lower doses of 1 and 2 µl/g DM had no effect on fermentation variables. This highlights that although *Trichoderma* enzymes can modulate the fermentation of low-quality feedstuffs *in vitro*, this is dose-dependent. As with pH stability, inappropriate dose rates of EE are frequently cited as potential explanations for when EE are ineffective in ruminants (Beauchemin et al., 2004, Meale et al., 2014).

Yang et al. (2022) measured the effects of seven doses of *Trichoderma reesei* derived xylanase and cellulase on *in vitro* and *in sacco* rumen fermentation of Faba bean silage (0, 0.25, 0.5, 0.75, 1, 1.25, and 1.5 ml/kg DM silage). 0.5 g and 7.5 g DM Faba bean silage was used in *in vitro* and *in sacco* incubations, respectively. Exogenous enzymes improved DMD and NDF degradation kinetics both *in vitro*, using Daisy[II] incubators, and *in sacco* following the protocol of

Ørskov et al. (1980). The soluble and potentially degradable NDF fraction of Faba bean silage was increased with increasing dose of EE whilst the undegradable fraction was linearly decreased. However, DMD was cubically improved, with the intermediate EE dose of 1 ml/kg DM silage having the highest digestibility of 56.54 %. This shows that higher doses of EE do not always exert greater effects on digestibility, further emphasizing the importance of characterizing optimal doses of EE. Notably, authors measured a robust correlation between *in vitro* and *in sacco* DMD ($r = 0.98$, $p < 0.01$) and NDF degradability ($r = 0.84$, $p < 0.01$); this demonstrates the utility of *in vitro* rumen simulations for screening the effects of enzymatic products on digestibility variables.

Romero et al. (2016) did not realize consistently beneficial lactation responses when supplementing two fermentation of *T. reesei* containing xylanase and cellulase + xylanase to sixty-six lactating Holstein cattle. Extracts were sprayed onto a dairy TMR and the xylanase treatment increased DM, OM and CP intakes. During the 10-week experiment, the treatments had limited effects on lactation. There was no improvement in milk solids or feed efficiency from either treatment, although during weeks 3, 6, and 9, cattle treated with xylanase had higher milk yield than controls. This transient response limits the evidence that the *Trichoderma* extract investigated could consistently improve milk yields on farm, although the authors suggest that the tendency ($p > 0.05$) of treatments to improve milk yield and solids is sufficient to infer enhanced lactation. A tendency of *Trichoderma* fermentation extract to improve lactational performance was also measured by Walker and Povey (2016). Fifty primiparous cows were recruited on a 12-week study to quantify the effect of pre-treating a TMR made up of grass silage, corn silage and concentrate with *Trichoderma* extract at 750 ml/T DM. During the first nine weeks of the study, milk yield tended to be higher in cows consuming the treatment TMR, however, in the last three weeks of the study milk production was significantly increased. This perhaps suggests that the effects of *Trichoderma* extract supplementation take several weeks to translate into production responses. Notably, the pregnancy rate of cows consuming *Trichoderma* extract was also 20 % higher than control

cattle, indicating potential benefits to fertility from supplementation. This effect requires further investigation and may be associated with the production of antimicrobials by *Trichoderma* (Zin and Badaluddin, 2020), potentially reducing the risk of conception limiting conditions, such as metritis and endometritis.

Fermentation products of *A. niger and A. oryzae* have also realized benefits for rumen fermentation kinetics and cattle performance. Tricarico and Dawson (2005) measured the effect of an *A. niger* extract with xylanase activities of 135 xylanase units (XU)/ml on VFA production from fescue hay, dry rolled corn, alfalfa pellets, oat by-product and a fescue hay-based TMR, in a batch culture rumen simulation using rumen fluid from a cannulated heifer. The *A. niger* extract increased tVFA production from the TMR, fescue hay, and alfalfa pellets, as well as increasing molar concentrations of acetate, propionate, butyrate, isobutyrate, and valerate from TMR, and acetate from fescue hay and alfalfa incubations. Notably, when the *A. niger* extract was combined with a *Trichoderma viride* extract containing endoglucanase activity of 5 carboxymethyl cellulase (CMCU)/ml, the acetate to propionate ratio in the TMR incubation was decreased, and tVFA production from the TMR, dry rolled corn, fescue hay, and alfalfa pellets was higher than when either extract was incubated on its own. This suggests an additive effect between fermentation products of *Aspergillus* and *Trichoderma*, where the combination of metabolites and enzymes in both products has increased tVFA production from substrate fermentation. Kong et al. (2021) treated a corn silage-based TMR, alfalfa hay, oat hay and corn silage with a fermentation extract from the co-cultivation of *A. oryzae* and *A. niger* (AOAN) to measure the effects of treatment on *in vitro* rumen fermentation. The AOAN decreased pH in all incubations, and increased tVFA production from all substrates apart from alfalfa hay. Acetate and the acetate to propionate ratio were increased in oat hay and alfalfa hay incubations. As in the study of Tricarico and Dawson (2005), the effects of this AOAN on VFA production were substrate specific. Kong et al. (2021) observed improvements in DM, ADF, and NDF digestibilities in all feed from AOAN treatment, except for ADF in alfalfa hay. This highlights the ability of AOAN to

enhance fibre digestibility across substrates. Microbiome analysis revealed that AOAN also influenced α and ß diversity; the prevalence of *Prevotella* was increased, and *Ruminococcus* decreased in TMR, alfalfa, and oat hay incubations, however, microbial diversity was unaffected in corn silage incubations. This indicates that fungal extracts could alter rumen fermentation through influencing microbial composition in the rumen or solely enhancing the digestibility of feeds with bioactive compounds present in the extract. Further investigation of these mechanisms would provide insight into how to maximise the digestibility enhancing capacity of AOAN.

Zhang et al. (2022) supplemented ten multiparous Holstein cows with 5 g/d *A. oryzae* extract (Nutri-Ferm; Nutri-AD International, Belgium) for five weeks. Cows fed *A. oryzae* extract had increased milk yield (1.5 kg/d) compared to controls, and higher yields and concentrations of milk protein and lactose, whilst milk fat production was unaffected. Rumen VFA production and proportions were unaffected as was rumen microbial diversity. Interestingly, the composition of the faecal microbiome was altered by treatment, with *A. oryzae* treated cows having an increased abundance of unclassified *Succinivibrio* spp. This genus is involved in the production of succinate and may be associated with increased caecal propionate production (Iqbal et al., 2018). Zhang et al. (2022) measured an increase in faecal propionate % and a decreased acetate: propionate ratio; as well as increased faecal pH in treatment cows, which should promote caecal epithelial health and enhance hindgut VFA absorption. The hindgut is an important site for VFA production and absorption, yet is often overlooked in cattle nutrition (Sanz-Fernandez et al., 2020). These results suggest that further investigation into the effects of *A. oryzae* in the cattle hindgut could be warranted.

When Oellermann et al. (1990) supplemented five non-lactating Holstein cows with five doses of *A. oryzae* extract (0, 1, 2, 4, 6 g/d) in a randomized block design study, supplementation had limited effects on rumen fermentation. A dose of 1 g/d decreased rumen pH, however, none of the doses influenced VFA production or the digestibilities of NDF, ADF and CP. Doses of 2, 4 and 6 g/d decreased the number of proteolytic bacteria in the rumen but did not increase

the proliferation of AF, cellulolytic bacteria, amylolytic bacteria, or protozoa. Arambel et al. (1987) suggests that *A. oryzae* can increase rumen proteolysis. So, perhaps, the population of proteolytic bacteria was suppressed by the endogenous proteolytic activity of *A. oryzae* in the experiment of Oellermann et al. (1990). However, this was not paired with increased CP digestibility so is not functionally advantageous. Conversely, when Wiedmeier et al. (1987) supplemented four non-lactating Holstein cows with 2.63 g/d *A. oryzae* extract (Amaferm®; BioZyme Inc, St Joseph, MO, USA), in a 4 × 4 Latin square design experiment, the digestibility of CP was increased, as was DM and hemicellulose digestibilities. Supplementation with *A. oryzae* extract increased the absolute and proportional amount of cellulolytic bacteria in rumen fluid, suggesting that *A. oryzae* increased structural carbohydrate hydrolysis and the proliferation of other fibrolytic microbes. This highlights the different effects that similar fungal products can have on the rumen microbiome; with Oellermann et al. (1990) observing an increase in proteolysis paired with a decrease in proteolytic microbes, and Wiedmeier et al. (1987) observing an increase in fibre degradation paired with an increase in fibrolytic microbes. Sucu et al. (2018) found that feeding primiparous and multiparous Holstein cows (n = 33) 15 g/d of the same *A. oryzae* extract as Wiedmeier et al. (1987) in a TMR consisting of alfalfa hay and steam-flaked corn, milk yield was increased by 3.1 kg/d compared to controls. *A. oryzae* treated cows achieved peak yield two weeks earlier than control cows, with average milk yields 4.5 kg/d higher in weeks three and four postpartum and no difference in volumetric concentrations of milk solids between groups. In this study, treatment administration began 21-d pre-calving, suggesting potential benefits from *A. oryzae* extract supplementation in the pre-fresh period that could lead to increased milk yields in the transition period. However, in a second experiment using 445 multiparous Holstein cows, *A. oryzae* extract supplementation (15 g/d) decreased milk yield by 0.8 kg/d. The DM intake of treatment cows was approximately 1 kg less per day than controls, which could indicate improved feed conversion efficiency (FCE), however, statistical analysis was not performed on these data. *A. oryzae* supplemented cows did have increased milk fat content, but ECM was unaffected. The forage to concentrate ratio (F:C) in TMRs offered to cattle may have led to different

responses in these experiments. In the first study F:C was 59:41 and in the second it was 44:56. Although both rations were corn and alfalfa based, the increased fibre content of the TMR in Experiment 1 may have had improved digestibility due to the fibrolytic activity of *A. oryzae*, leading to increased energy availability and consequently increased milk yield. These two experiments demonstrate that even when the same dose of the same *A. oryzae* extract is administered to cows on similar diets, very different responses from treatment can occur. This hinders the use of fungal fermentation products in cattle, as inconsistent outcomes do not encourage industry uptake. The effects of the *A. oryzae* extract, Amaferm®, in dairy cattle have been assessed using meta-analysis. The results of the analysis of 26 studies by Cantet et al. (2025), found decreases in milk fat and protein, and increases in milk yield, ECM, DMI, rumen pH and acetate: propionate in the rumen. This demonstrates the power of meta-analysis for consolidating and analysing the effects of treatments where individual studies present inconsistencies. These analyses can increase the industry acceptance of such products, by providing strong evidence of the effects likely to be observed on farm.

There is limited literature examining the use of *Aspergillus* products in young cattle. Anil et al. (2022) measured the effect of two *Aspergillus niger* fermentation extracts with xylanase and cellulase activities of 8000 and 16000 U/kg DM and 12000 and 24000 U/kg DM, respectively, in 7-month-old crossbred Jersey heifers. Heifers were fed a TMR consisting of concentrates, chaffed rice paddy straw and oat fodder, and both treatments increased TMR digestibility, feed intake and FCE. Conversely, when Vaz-Ramírez et al. (2021) supplemented pre-weaned Jersey and Holstein calves 2 g/d of *A. niger* and *A. oryzae* fermentation product, no effects on growth or health status were measured. It is possible that aerobic fungi and their fermentation products could enhance the performance of calves through increased fibre digestion and rumen development. However, there is sparse evidence to validate or refute this hypothesis and the topic requires further investigation.

2.6 Yeasts

The use of yeast additives in cattle has been rigorously researched and reviewed (Beev et al., 2007, Shurson, 2018, Cangiano et al., 2020, Baker et al., 2022), and the volume of work is such that comprehensively evaluating all the literature examining yeast supplementation in dairy cattle is beyond the scope of this paper; as such we will discuss the principles of adding yeast to cattle diets, and associated advantages and challenges. *S. cerevisiae* products can be purely live cells, cultures containing live or inactive cells and fermentation metabolites, nutritional by-products from brewing, or cell components (Shurson, 2018). Viable yeast additives are sometimes beneficial in the rumens of mature cattle and calves, where the probiotic effects of *S. cerevisiae* can stimulate the growth of cellulolytic and lactate-utilizing bacteria (Baker et al., 2022). Propagation of these microbial communities often leads to enhanced fibre digestion, increased butyrate production, a lower acetate to propionate ratio, and increased pH stability in the rumen; which can cause beneficial responses in DMI, FCE, rumen development, lactation, growth, reduced output of CH_4 and animal health (Beev et al., 2007, Baker et al., 2022). *S. cerevisiae* is a facultative anaerobe, so can de-oxygenate the rumen, producing a more favourable environment for anaerobic microbe growth and fibre degradation (Baker et al., 2022). This is particularly important in calves, where transitioning from liquid to solid feed relies on microbial and histological development in the rumen. Yeast supplementation can lead to improvements in feed intake and growth in calves. However, of seventeen studies reviewed by Cangiano et al. (2020) examining the effects of yeast culture or live yeast in calves, only five studies realized an increase in weight gain (Lesmeister et al., 2004, Galvão et al., 2005, Zhou et al., 2009, Brewer et al., 2014, Villot et al., 2019), and no studies measured an effect on FCE. The effects of yeast supplementation on GI tract development were much more consistent: of six studies that measured the effect of treatment on gut, haematological, or microbiome variables, five found a positive effect (Kaldmäe et al., 2008, Hučko et al., 2009, Brewer et al., 2014, Xiao et al., 2016, Harris et al., 2017), but only Brewer et al. (2014) also realized an increase in

growth. This indicates that *S. cerevisiae* does not always improve primary response variables in calves but reliably promotes health and gut development.

Similarly to filamentous fungal additives, the effects of yeast cultures can be probiotic, prebiotic and/or nutritional. Cultures contain live or inactive cells, culture media, and compounds produced during fermentation, including organic acids, peptides, enzymes, esters, and alcohols. The profile of compounds produced is dependent on *S. cerevisiae* strain, the composition of the culture media and the conditions under which fermentation takes place (Shurson, 2018). *S. cerevisiae* is rich in amino acids, lipids, minerals and vitamins that can be utilized directly by cattle, or by rumen microbes to produce VFAs and microbial protein (Baker et al., 2022). The bioavailability of nutrients in these products can be highly variable and can be reduced by the formation of Maillard products from high drying temperatures (Shurson, 2018). Similarly to products containing only active yeast, yeast cultures can enhance the abundance and activities of beneficial microbes in the rumen, leading to improved FCE, productivity and animal health. However, because the composition of different yeast cultures can be varied depending on culture conditions, yeast strain and viability, there can be differences in animal responses to supplementation (Beev et al., 2007).

A meta-analysis performed by Ondarza et al. (2010) demonstrated that live yeast supplementation is associated with increased milk production in cattle, but that this was not associated with increased DMI, suggesting that improved lactation could be caused by enhanced rumen function and FCE rather than feed intake. As well as scavenging oxygen and improving fibre degradation, *S. cerevisiae* can prevent lactate accumulation in the rumen by outcompeting lactate producing microbes for fermentable sugars (Ding et al., 2014) and stimulating the growth and activities of lactate metabolizing bacteria (Fonty and Chaucheyras-Durand, 2006). This leads to increased pH and pH stability in the rumen, limiting the risk of acidosis which is highly prevalent in cattle fed large quantities of starch; although some studies have found that live *S. cerevisiae* does not affect rumen pH (Ambriz-Vilchis et al., 2017). As with studies investigating aerobic and anaerobic fungal feed additives, variation from heterogenous yeast product composition can be compounded by dietary, environmental, and animal

differences in experiments. Given the abundance of research examining yeast in cattle, the strength of evidence for the efficacy of specific cultures can sometimes be quantified through meta-analysis. This highlights how consolidation of existing research on individual fungal additives can provide greater insights into treatment effects without the need for further *in vitro* or *in vivo* experimentation.

Yeast cell walls contain MOS and ß-Glucans that can have beneficial effects on immunity, gut health, and productivity (Broadway et al., 2015). Cell wall contents can be extracted from fragmented yeast cells or be a component of hydrolysed yeast cultures. MOS and ß-Glucans are mycotoxin binders, can regulate the activation of the hypothalamic-pituitary-adrenal axis, modulate the immune system by interacting with immune cells, and enhance the phagocytosis of pathogens by leucocytes (Pedro et al., 2021). As biological response modifiers, MOS and ß-Glucans can affect the functionality of macrophages and neutrophils which can lead to enhanced production of pro-inflammatory cytokines when animals are challenged by stress (Broadway et al., 2015). Dairy cattle encounter stress throughout their lives, including birth, weaning, heat stress, social stress, disease and calving. Improved humoral and innate immunity from MOS and ß-Glucan supplementation can increase the capacity of cattle to tolerate and recover from environmental and disease stressors (Ma et al., 2020). In calves, yeast cell walls can enhance GI tract histology, increasing the length and width of papillae in the rumen due to increased butyrate production from *Butyrivibrio* spp., for which yeast and yeast cell walls provide a more favourable environment for growth (Ma et al., 2020). Yeast culture supplementation has also led to increased villus height to crypt depth ratio in the duodenum, jejunum and ileum in neonatal calves (Xiao et al., 2016). The actions of yeast cultures and cell walls that lead to improved small intestine histology are not well defined, but are likely due to favourable manipulation of the gut microbiome, engulfing pathogens and mycotoxins and inhibiting their ability to bind to the epithelium, activating cytokine responses in mucosa, and improving epithelial barrier function through increased expression of tight junction proteins (Xiao et al., 2016, Ma et al., 2020, Cangiano et al., 2020). Considerably less research has

been performed to investigate pure cell wall additives compared to yeast cultures and active yeast. When yeast cultures are hydrolysed to disrupt cells, they should be able to elicit the same benefits as cell wall products but have the advantage of containing other cell components and fermentation products that can also cause beneficial responses. Further research is required to define optimal inclusion rates and dietary interactions that can hinder the efficacy of yeast products, but as highlighted by the large volume of research that has already been performed, yeast supplementation typically improves cow and calf health, which is often paired with improvements in productivity.

2.7 Conclusion

This review highlights the potential of fungal feed additives to improve the productivity of dairy cattle through enhanced feed digestibility, microbial growth, gut health and development, and immunity. Anaerobic fungi have elite fibre degrading capabilities but the utility of these additives in dairy cattle is limited by oxygen vulnerability; research to overcome this via encapsulation or further investigation into the resting stage of the AF lifecycle could increase the accessibility of these additives to industry. Aerobic fungal products can be rich in enzymes, bioactive metabolites, and nutrients, which can enhance rumen fermentation variables, productivity, and favourably manipulate the rumen microbiome in cattle. However, inconsistent responses to treatments limit their uptake in industry and further research is required to define optimum inclusion rates, substrate specificity and interactions with endogenous microbes and co-cultivation products. Notably, most of the published literature examining AF and aerobic fungal additives comes from *in vitro* experimentation; a greater number of *in vivo* studies are required to define additive interactions in animal models, and meta-analyses of current literature could provide further insights into product efficacy. Conversely, yeast and yeast components have been researched comprehensively *in vitro, in vivo* and in meta-analyses. Investigation into potential synergies between yeasts and other fungal additives could increase the already broad range of benefits that can occur from yeast supplementation in cattle. Across all fungal additive types, existing research demonstrates that

achieving consistent responses from treatments is often challenging, with inconsistent responses limiting industry uptake. Defining fungal additive interactions at dietary, cellular, and microbiome levels will increase the utility of these products on farm by informing optimal conditions for efficacy.

Chapter 3. The effects of a product of the solid-state fermentation of *Aspergillus niger* on the *in vitro* rumen fermentation kinetics of rations fed to dairy cattle

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3.1 Abstract

The aim of this study was to determine the effects of incubating different doses of Synergen® (0, 0.5, 2, 5 mg/g substrate), a product of the solid-state fermentation of *Aspergillus niger* (ANP) containing residual enzyme activities, with two total mixed rations (TMR) formulated for dairy cattle, on the kinetics and end products of *in vitro* rumen fermentation. The inclusion of fungal fermentation products in cattle rations has improved digestibility measures and production performance and may offer an avenue to improve the sustainability of ruminant production. In this study, a dose-responsive effect was observed for gas production from a high starch TMR over a 72-h incubation; doses of 2 and 5 mg ANP/g substrate significantly increasing gas production by 33 ml (p = 0.024) and 32.9 ml (p = 0.025), respectively, compared to controls. However, no effect of treatment was observed on volatile fatty acid composition at the end of incubations. ANP treatment had no effect on the fermentation kinetics of a low starch TMR, suggesting that the dominant effect of treatment was amylolytic. Further research into the effects of ANP in cattle models is required to explore the utility of the product on farm.

Keywords: Ruminant, digestibility enhancer, feed efficiency, fungal fermentation product, *in vitro* gas production

3.2 Implications

This study used a rumen simulation technique to measure effects of a commercially available feed material (Synergen®), derived from the solid-state fermentation of a fungus, on the digestibility of two rations formulated for dairy cattle. Digestibility was measured by gas production, and doses of 2 and 5 mg/g substrate, equivalent to feeding cattle 10 or 25 g/d, significantly increased the gas produced from a high starch ration but had no effect on a low starch ration. This suggests that Synergen® can enhance the digestibility of high starch dairy rations, although validation in animal models is required.

3.3 Introduction

Cattle play a crucial role in the global food system, converting non-human edible feeds into highly nutritious human edible protein. However, inefficient digestion of forages limits the sustainability of cattle production, with plant cell wall digestibility estimated to be 65 % under ideal conditions in the rumen (Van Soest, 1994). Inefficient utilisation of forage is undesirable for cattle productivity, as well as contributing to increased greenhouse gas emissions intensity (GHG EI), an issue for which the ruminant sector is under increasing scrutiny (Arndt et al., 2022). Thus, exploration of strategies that could enhance the digestion of cattle feedstuffs are valuable across environmental and economic agendas. The use of fermentation products of *Aspergillus* spp. to enhance the digestibility of cattle feeds has realised positive outcomes for *in vitro* rumen fermentation (Tricarico and Dawson, 2005, Kong et al., 2021) and in digestibility and production responses in animal studies (Caton et al., 1993, Tricarico et al., 2005, Sosa et al., 2022). The findings of these studies demonstrate that the inclusion of the fermentation products of *Aspergillus* spp. can increase the digestibility of feeds and favourably manipulate the products of fermentation, leading to increased feed intake and production. Fungi digest plant matter extracellularly; so, when cultivated on a substrate, produce a suite of substrate specific enzymes and non-enzyme bioactive compounds for digestion

(Li et al., 2020). It is widely accepted that fungal fermentation products can increase feed digestibility by catalysing the hydrolysis of substrates. However, there is evidence that they may work synergistically with endogenous enzymes in the rumen and stimulate the rumen microbiome towards more efficient fermentation pathways, leading to decreased GHG EI and increased volatile fatty acid (VFA) and microbial protein production (Meale et al., 2014, Sun et al., 2017). This suggests that beneficial responses from the addition of fungal fermentation products to ruminant diets are multifactorial. However, responses to supplementation with *Aspergillus* spp. fermentation products are not always consistent, with some studies realising limited or no effects from treatments (Sievert and Shaver, 1993, Giraldo et al., 2007B). Variable responses have been attributed to the stability and substrate specificity of fermentation products, which has hindered their widescale industry application (Beauchemin et al., 2004).

Synergen® (Alltech Inc., Kentucky, US) is a commercially available product of the solid-state fermentation (SSF) of *Aspergillus niger* (ANP), containing residual enzyme activities. SSF is a method of biotransformation, where microorganisms are propagated on an organic substrate in the absence of free-flowing water, to produce desirable fermentation products such as organic acids, enzymes, alkaloids, and plant growth factors (Pandey, 2003). The goal of the present study was to measure the effect of a range of doses of Synergen® on gas production (GP), rumen fermentation kinetics, and DM disappearance (DMD) of two total mixed rations (TMR) with varying levels of starch, formulated to meet the dietary requirements of dairy cattle, using an *in vitro* rumen simulation technique (ANKOM, 2018), with a view to determining the doses and diets that should be used for animal trials. Although *in vitro* results do not always translate into *in vivo* effects, they do allow for the preliminary assessment of products without the use of large numbers of research animals, which is ethically and economically undesirable.

3.4 Materials and methods

3.4.1 Study design

3.4.1.1 Animals and treatments

Four cannulated, non-lactating Jersey cows, on a partial mixed ration consisting of barley straw (75%) and grass silage (25%), and 350 g/d concentrates comprising of 150 g steam-flaked barley and 200 g soaked sugar beet, were used as digesta donors for *in vitro* GP. Cows were randomly allocated to control or treatment groups, with each of the treatment cows fed 5 g/d ANP mixed with concentrates for 21-d of adaption prior to digesta collection. The GP trial followed the protocol of Theodorou et al. (1994) and measured the effect of four doses of ANP: 0, 0.5, 2, 5 mg/g substrate, on a maize-based and a grass silage-based TMR formulated for dairy cattle (Table 3-1 and Table 3-2). *In vitro* doses would be approximately equivalent to feeding cattle 0, 2.5, 10 and 25 g/d ANP and were selected to represent a range of inclusion rates that could be practical to incorporate into farm rations. TMR were dried at 55 °C for 48-h and DM was calculated using the method of Goering and Van Soest (1970). Substrates were ground to pass through a 2 mm sieve (Retsch Cyclone, Germany), and 1 g DM samples were weighed into 40 ANKOM *in-situ* bags (5 × 10 cm, 50 µm porosity).

Table 3-1. Feed composition of total mixed rations used in rumen fermentation simulation to measure the effects of Synergen in cattle.

	High starch ration (% as fed)	Low starch ration (% as fed)
Grass silage	76.1	24.2
Maize silage	-	37.3
Lucerne silage	-	7.4
Wheat straw	-	1.9
Mineral blend	-	0.7
Wheat	-	6.5
Dairy concentrates	22.4	17.3
Molasses	1.5	4.7

Table 3-2. Chemical composition of total mixed rations used in rumen fermentation simulation to measure the effects of Synergen in cattle.

	High starch ration	Low starch ration
DM (%)	35.6	36.1
Organic matter (%)	91.2	92.7
NDF (%)	35.3	38.1
ADF (%)	20.8	19
CP (%)	16	16.4
Starch (%)	28.8	19.6
Metabolizable energy (MJ/kg)	10.3	10.1

3.4.1.2 *In vitro* gas production

In vitro GP was performed using the ANKOM^{RF} Gas Production System (ANKOM Technology, USA). This system uses gas produced as a measure of digestibility; real time monitoring of gas pressure allows for the fermentation kinetics of feeds and any impact of treatments to be modelled. Each dose of ANP was incubated in quadruplicate with 1 g DM of each TMR, buffer solution, and microbial inoculum prepared from the digesta of two cows adapted to ANP. As controls, four replicates of each TMR were incubated with no ANP and with microbial inoculum prepared from digesta of two cows not adapted to ANP; eight blank bottles were incubated containing buffer and no substrate, half of which contained microbial inoculum from ANP adapted cows, the rest contained microbial inoculum from control cows.

Samples were incubated in 250 ml glass bottles with 89 ml of digestion medium, the composition of which is described by Theodorou et al. (1994), and 10 ml of microbial inoculum. Inoculum was prepared by collecting 1 l of digesta from the ventral sac of the rumen of each cow, immediately prior to the morning feed. Under anaerobic conditions, digesta was strained through a double layer of cheesecloth; the solid component was then homogenised with an equal measure of digestion medium and strained once more. The strained fluid from adapted cows was pooled and mixed on a hot plate, as were the fluids from the control cows. After inoculation, bottles were gassed with CO_2 before being sealed with ANKOM^{RF} pressure modules and placed in water baths set to 39°C for a 72-h incubation. The ANKOM^{RF} system measures cumulative gas pressure in each bottle at 10-min intervals; gas is released from modules when headspace pressure reaches 0.75 psi above atmospheric pressure, with data readings transmitted wirelessly to a computer.

3.4.1.3 Parameters

Following 72-h of incubation, gas pressure recordings were saved into an Excel spreadsheet and ANKOM^{RF} modules were removed from bottles. The pH of fermentation fluid was measured (TRUEscience Smart Cap, UK) and a 10 ml

aliquot of fermentation fluid from each bottle was stored with 0.5 ml orthophosphoric acid prior to gas chromatography for VFA analysis. *In-situ* bags were removed from bottles and dried at 55 °C for 24 h before weighing.

3.4.2 Statistical analysis of data

Dry matter disappearance of substrates was calculated using the following equation:

$$DM\ recovered = (post\ incubation\ substrate + in\ situe\ bag\ weight) - bag\ weight$$

$$DMD = pre - incubation\ substrate - DM\ recovered$$

Gas production data were converted from psi into moles using the Ideal Gas Law:

$$n = \frac{PV}{RT}$$

n = gas produced (mol)

P = pressure (kPA)

V = head-space volume in each bottle (L)

R = gas constant (8.314472L·kPa·K^{-1} mol^{-1})

T = temperature (K)

Gas production in moles was converted to ml using Avogadro's Law:

$$y = n \times 22.4 \times 1000$$

y = gas produced (ml)

n = gas produced (mols)

Where 22.4 is the volume (l) that 1 mole of gas will occupy under standard conditions (273.15 °K and 101.325 kPa).

Volatile fatty acid proportions and concentrations were measured using gas chromatography, following the procedures described by Erwin et al. (1961).

One-way ANOVA in R (R Core Team, 2024) was used to analyse the effect of ANP dose on total GP, parameters of GP kinetics, DMD, pH, total VFA, acetate: propionate ratio, acetate: total VFA, and molar concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. All data sets passed Shaprio-wilk normality tests and Levene's tests for homogeneity of variance ($p > 0.05$). Tukey's Honest Significant Difference post hoc test was used to assess differences between means, with significance declared at $p < 0.05$. Gasfit system software (Texas A&M) was used to identify the optimal model to describe the GP kinetic profiles from the ANKOM^{RF} modules. Gasfit uses an R script to trial different nonlinear functions on GP profiles, with the best model selected based on the lowest sum of squares error.

3.5 Results

The GP profiles of high and low starch TMR incubated with different doses of ANP are displayed in Fig. 3-1 and Fig. 3-2, respectively. In high starch incubations, the 0 mg/g treatment group incubated with inoculum derived from cattle not adapted to ANP increased the GP to 97 ml after 72-h; the 0 mg/g treatment group incubated with ANP adapted inoculum increased the GP to 110 ml after 72-h; the 0.5 mg/g treatment group increased the GP to 114.5 ml after 72-h; the 2 mg/g treatment group increased the GP to 130 ml after 72-h and the 5 mg/g treatment group increased the GP to 129.9 ml after 72-h. The increase in GP in a dose responsive manner observed for the high starch TMR was not observed in low starch TMR incubations. In low starch incubations (Fig. 3-2), the 0 mg/g treatment group incubated with inoculum derived from cattle not adapted to ANP increased the GP to 119.8 ml after 72-h; the 0 mg/g treatment group incubated with ANP adapted inoculum increased the GP to 120 ml after 72-h; the 0.5 mg/g treatment group increased the GP to 130.5 ml after 72-h; the 2 mg/g treatment group increased the GP to 120.5 ml after 72-h and the 5 mg/g treatment group increased the GP to 112.6 ml after 72-h. Table 3-3 and Table 3-4 display the effect of ANP dose on total GP, DMD, and pH of high and low starch TMR incubations, respectively. High starch TMR incubations treated with doses

of 2 and 5 mg ANP had total GP values 34 % (p = 0.024) and 33.9 % (p = 0.025) greater than the 0 mg non-adapted inoculum control group, respectively. Non-significant numerical increases in DMD were observed in high starch incubations, with the 2 mg ANP treatment group having a mean DMD 13.8 % higher than the 0 mg non-adapted inoculum control group. In low starch incubations, there was no significant difference in total GP, DMD, and pH between control and treatment groups, however, the 0.5 mg ANP treatment group had significantly higher DMD than the 5 mg ANP group, 84 versus 76 %. For the GP data produced from this experiment, Gasfit determined that the logistic nonlinear function with two pools and a single lag time, was most appropriate for modelling, with an R^2 > 98.5 for all modules. This model was developed by Schofield et al. (1994) using the premise that feedstuffs are composed of two pools of components: a rapidly degradable fraction and a slowly degradable fraction. The rapidly degradable fraction is composed of non-fibrous carbohydrates, non-protein nitrogen, and soluble amino acids. The slowly degradable fraction is made up of true protein and structural carbohydrates. Table 3-3 and Table 3-4 present the GP kinetic parameters of the Schofield et al. (1994) model for both TMR incubated with different doses of ANP. No significant differences were observed between treatments for any parameter. The Cumulative Gas Production Equation used by the Schofield et al. (1994) model is:

$$Y = \left(\frac{a}{1 + \exp\left(2 + 4 \times b \times (c - t)\right)}\right) + \left(\frac{d}{1 + \exp\left(2 + 4 \times e \times (c - t)\right)}\right)$$

Y = cumulative gas produced (ml)

a = volume of gas produced from the rapidly degradable fraction (ml)

b = degradation rate of the rapidly degradable fraction (h^{-1})

c = lag time (h)

t = time (h)

d = volume of gas produced from the slowly degradable fraction (ml)

e = degradation rate of the slowly degradable fraction (h^{-1})

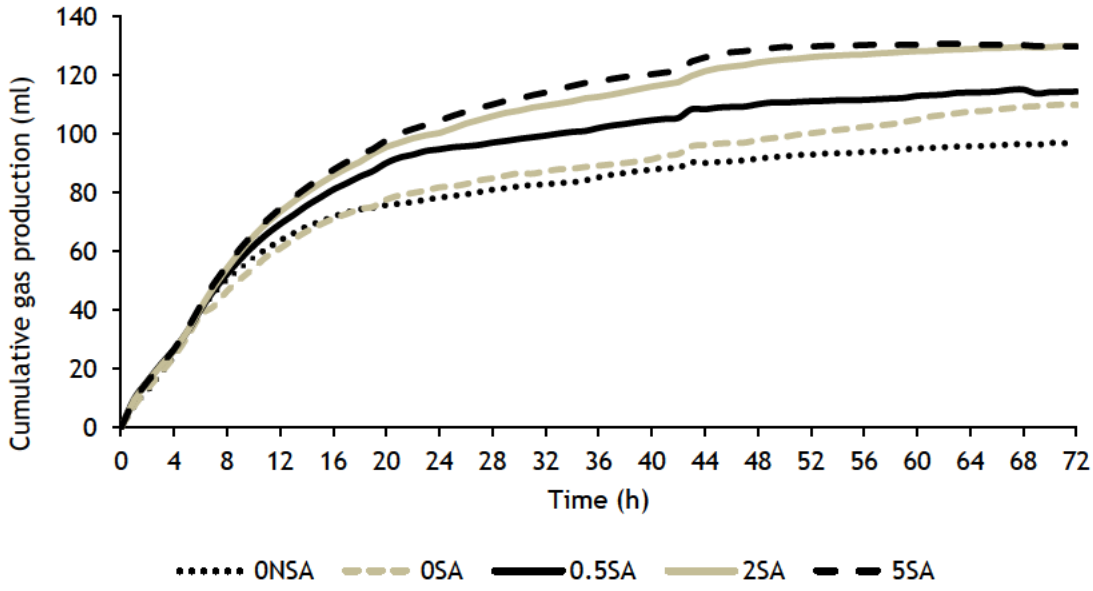


Figure 3-1. Gas production curves of 1 g DM high starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle (ANKOM, 2018).Treatments: 0NSA = 0 mg Synergen® incubated with inoculum from donor cattle not adapted to Synergen®; 0SA = 0 mg Synergen® incubated with Synergen® adapted inoculum; 0.5SA = 0.5 mg Synergen® incubated with Synergen® adapted inoculum; 2SA = 2 mg Synergen® incubated with Synergen® adapted inoculum; 5SA = 5 mg Synergen® incubated with Synergen® adapted inoculum.

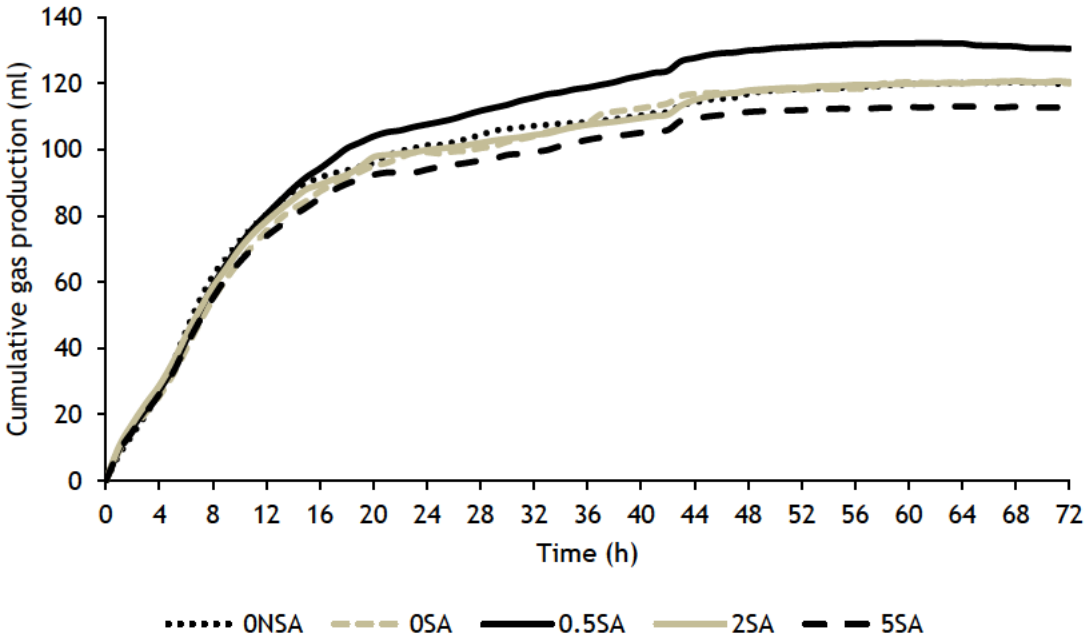


Figure 3-2. Gas production curves of 1 g DM low starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle (ANKOM, 2018). Treatments: 0NSA = 0 mg Synergen® incubated with inoculum from donor cattle not adapted to Synergen®; 0SA = 0 mg Synergen® incubated with Synergen® adapted inoculum; 0.5SA = 0.5 mg Synergen® incubated with Synergen® adapted inoculum; 2SA = 2 mg Synergen® incubated with Synergen® adapted inoculum; 5SA = 5 mg Synergen® incubated with Synergen® adapted inoculum.

Table 3-3. Total gas production, gas production kinetic parameters, DM disappearance, and digestion medium pH, of 1 g DM high starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle.

	Synergen dose (mg) and rumen inoculum source						
Parameter	0 NSA[1]	0 SA[2]	0.5 SA	2 SA	5 SA	Pooled SE	*P*-value
DMD (g)	0.72	0.78	0.77	0.82	0.8	0.013	0.18
pH	6.73	6.74	6.71	6.70	6.71	0.011	0.84
Total GP (ml)	97[a]	110[ab]	114.5[ab]	130[b]	129.9[b]	3.93	0.016
a (ml)	59.4	54.9	69.6	67.3	66.9	2.47	0.29
b (h^{-1})	0.112	0.101	0.088	0.099	0.099	0.0033	0.26
c (h)	1.49	1.84	1.04	1.73	1.74	0.11	0.12
d (ml)	42.8	73.4	46.2	64.3	64.7	4.68	0.17
e (h^{-1})	0.021	0.017	0.022	0.023	0.025	0.0016	0.62

Abbreviations: NSA = non-Synergen® adapted inoculum; SA = Synergen® adapted inoculum; DMD dry matter disappearance; GP = gas production; a = gas derived from the rapidly degradable fraction of substrate; b = degradation rate of the rapidly degradable fraction; c = lag time of incubation; d = gas derived from the slowly degradable fraction of substrate; e = degradation rate of the slowly degradable fraction. [1] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows not adapted to Synergen®. [2] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows adapted to Synergen® for 21 days. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

Table 3-4. Total gas production, gas production kinetic parameters, dry matter disappearance, and digestion medium pH, of 1 g DM low starch total mixed ration incubated with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle.

	Synergen dose (mg) and rumen inoculum source						
Parameter	0 NSA[1]	0 SA[2]	0.5 SA	2 SA	5SA	Pooled SE	*P*-value
DMD (g)	0.79	0.81	0.84[a]	0.8	0.76[b]	0.009	0.062
pH	6.64	6.7	6.66	6.7	6.73	0.012	0.12
Total GP (ml)	119.8	120	130.5	120.5	112.6	2.98	0.49
a (ml)	77.4	73.3	76.9	78	73	1.3	0.66
b (h^{-1})	0.113	0.098	0.098	0.094	0.099	0.0024	0.12
c (h)	1.94	1.78	1.86	1.29	1.53	0.096	0.18
d (ml)	46.3	48.8	56.6	46.2	41.3	2.86	0.59
e (h^{-1})	0.022	0.022	0.024	0.019	0.022	0.0011	0.71

Abbreviations: NSA = non-Synergen® adapted inoculum; SA = Synergen® adapted inoculum; DMD = dry matter disappearance; GP = gas production; a = gas derived from the rapidly degradable fraction of substrate; b = degradation rate of the rapidly degradable fraction; c = lag time of incubation; d = gas derived from the slowly degradable fraction of substrate; e = degradation rate of the slowly degradable fraction. [1] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows not adapted to Synergen®. [2] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows adapted to Synergen® for 21 days. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

Table 3-5 and Table 3-6 display the VFA profiles of fermentation fluid collected vessels after different doses of ANP were incubated with both TMR for 72-h. No significant differences in VFA concentrations or proportions were observed between treatments for either TMR.

Table 3-5. Volatile fatty acid profiles of fermentation fluid from incubating 1 g DM high starch total mixed ration with different doses of Synergen for 72 hours, in a rumen fermentation simulation for cattle.

Parameter	Synergen dose (mg) and rumen inoculum source					Pooled SE	*P*-value
	0 NSA[1]	0 SA[2]	0.5 SA	2 SA	5SA		
Acetate (mM)	25.77	24.33	22.44	24.3	19.69	1.22	0.607
Propionate(mM)	15.69	14.02	13.11	14.39	10.2	1.06	0.601
Isobutyrate (mM)	0.52	0.51	0.52	0.58	0.41	0.032	0.625
Butyrate (mM)	4.9	4.65	4.46	5.17	3.6	0.34	0.691
Isovalerate (mM)	0.79	0.74	0.69	0.82	0.56	0.053	0.63
Valerate (mM)	0.82	0.8	0.74	0.85	0.61	0.052	0.683
tVFA (mM)	48.49	45.05	41.95	46.12	35.09	2.74	0.627
Ace: Prop	1.71	1.82	1.75	1.74	2.24	0.099	0.434
Acetate: tVFA	53.68	54.89	53.96	53.25	58.48	0.99	0.493

Abbreviations: NSA = non-Synergen® adapted inoculum; SA = Synergen® adapted inoculum; tVFA total volatile fatty acids; Ace: Prop = acetate: propionate. [1] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows not adapted to Synergen®. [2] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows adapted to Synergen® for 21 days. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

Table 3-6. Volatile fatty acid profiles of fermentation fluid from incubating 1 g DM low starch total mixed ration with different doses of Synergen. for 72 hours, in a rumen fermentation simulation for cattle.

Parameter	Synergen dose (mg) and rumen inoculum source					Pooled SE	*P*-value
	0 NSA[1]	0 SA[2]	0.5 SA	2 SA	5SA		
Acetate (mM)	21.98	23.23	22.89	21	19.99	1.3	0.947
Propionate(mM)	13.58	14.48	13.94	12.4	11.26	1.3	0.954
Isobutyrate (mM)	0.49	0.5	0.5	0.49	0.44	0.039	0.987
Butyrate (mM)	3.8	4.49	4.73	4.07	3.43	0.38	0.855
Isovalerate (mM)	0.67	0.7	0.72	0.66	0.57	0.063	0.964
Valerate (mM)	0.78	0.85	0.87	0.78	0.71	0.066	0.957
tVFA (mM)	41.3	44.26	43.65	39.39	36.39	3.13	0.948
Ace: Prop	1.79	1.89	1.87	1.79	2	0.12	0.985
Acetate: tVFA	54.93	54.83	54.41	54.1	56.76	1.3	0.98

Abbreviations: NSA = non-Synergen® adapted inoculum; SA = Synergen® adapted inoculum; tVFA = total volatile fatty acids; Ace: Prop = acetate: propionate. [1] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows not adapted to Synergen®.[2] Microbial inoculum used in the rumen fermentation simulation was derived from two Jersey cows adapted to Synergen® for 21 days. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

3.6 Discussion

The results of this experiment provide evidence that ANP can significantly increase *in vitro* digestibility of high-starch, grass-silage-based rations, when included at doses of 2 and 5 mg/g substrate. However, treatments did not alter the quantities or proportions of the end products of fermentation. No significant effect was found for any of the variables measured when ANP was incubated with a low-starch, maize-based TMR; suggesting that the dominant effect of ANP is amylolytic. Considering the association between ANP and the DMD and GP of the high starch TMR, although the differences in DMD were not significant, they followed the pattern that would be expected, given the increase in GP: the fermentation of more feed substrate would lead to a greater volume of GP, and a greater disappearance of DM (Theodorou et al., 1994). This indicates that *in vitro*, ANP enhances the capacity of rumen microbes to hydrolyse substrates, fitting with a proposed mode of action for fungal fermentation products, whereby nutrient availability is improved through catalysing the hydrolysis of plant polysaccharides (Beauchemin et al., 2003). Increased gas production is usually associated with increased VFA production (Doane et al., 1997), yet there was no detectable association between VFA concentrations and GP in this experiment. In contrast to our study, Giraldo et al. (2007B) reported a significant increase in GP when treating low quality forages with a fibrolytic *A. niger* fermentation product *in vitro*, however increased GP was associated with increased propionate and total VFA production. Unlike the straw, hay, and corn stover used in the study of Giraldo et al. (2007B), both TMR trialled in the present study were highly digestible, which may have limited the ability of ANP to increase their hydrolysis. However, Giraldo et al. (2007A) trialled the effects of the same *A. niger* fermentation product on a 60:40 forage: concentrate mix, on *in vitro* rumen fermentation, and although no increases in total GP were observed, treatments did increase acetate, propionate, butyrate, and total VFA production. Tricarico and Dawson (2005) measured the effect of an *A. niger* fermentation product on *in vitro* ruminal VFA production from hay, corn, alfalfa, and oat byproduct, and found that supplementation significantly increased VFA

production from substrates but did not alter the acetate to propionate ratio. Similar to the present study, Kong et al. (2021) found that the influence of a fermentation product from the mixed cultivation of *A. niger* and *A. oryzae* (AOAN) on *in vitro* rumen fermentation, was diet specific. In their trial, gas production was significantly increased from treating corn silage, oat hay, alfalfa hay, and a corn silage-based TMR, with 30 mg AOAN / 500 mg substrate. Substrates treated with AOAN fermentation extract had significantly increased digestibilities of DM, NDF, and ADF, and the digestibility of starch was significantly increased in corn silage. Notably, Kong et al. (2021) performed microbiome analysis and found that AOAN affected α and ß diversity in TMR, oat hay, and alfalfa silage incubations, with increased *Prevotella* and decreased *Ruminococcus* abundance, but treatment did not affect microbial diversity in corn silage. This suggests that the way *Aspergillus* fermentation products act is diet specific; either influencing the microbial diversity which can lead to positive outcomes for ruminal fermentation, or in the case of corn silage, acting purely as a digestibility enhancer without affecting microbial composition. The lack of influence that ANP treatment had on the end products of fermentation in the present study is not in line w ith previous work examining the effect of *A. niger* products on *in vitro* rumen fermentation. Although *in vitro* experimentation is a valuable way of screening the type of products used in the current study, the conditions *in vitro* differ from those in the rumen; rumination, nutrient absorption, and rumen motility are not simulated in the ANKOM system, and as such, drawing conclusions from results without *in vivo* validation is not recommended (McAllister et al., 2001).

In the present study, analysis of variance between the parameters of the Schofield et al. (1994) model did not find any significant differences between treatments for either TMR. Modelling gas curves is a useful tool to understand which fractions of feeds are being affected by a treatment, and at what rate. Modelling estimated that the slowly degradable fraction of the high starch TMR produced 50 % more gas at ANP inclusion rates of 2 and 5 mg than the control (parameter d, Table 3-3). This suggests that ANP is potentially increasing the digestibility of the structural carbohydrates and true protein present in the

slowly degradable fraction of the high starch TMR (Schofield et al., 1994). An increase in the digestibility of the slowly degradable fraction would be indicative of secondary fibrolytic activity and is in line with previous research investigating the effect of fermentation products of *A. niger*, where *in vitro* and *in sacco* fermentation of the slowly degradable fraction of forages was significantly increased by treatments (Gallardo et al., 2010, Kong et al., 2021). One of the substrates trialled by Gallardo et al. (2010) was corn stover, similar to the low starch TMR in the present study, and no effect of any *A. niger* treatment was observed on its digestibility.

3.7 Conclusion

This preliminary experiment to examine the effects of Synergen® on rumen fermentation kinetics has suggested that the effect of Synergen® is likely predominantly amylolytic, and that an inclusion rate of 2 mg/g substrate is sufficient to significantly increase the *in vitro* digestibility of a high starch TMR. This experiment provided evidence against the null hypothesis that the inclusion of different doses of Synergen® with TMR, in an *in vitro* rumen simulation, will have no effect on the digestibility characteristics of the feed. These results suggest that this ANP could have the potential to act as a digestibility enhancer in cattle rations, which might have beneficial outcomes for production responses, feed efficiency and the environment. The present study is limited by differences in *in vitro* conditions and those found in animals. As such, further experimentation to model the effects of Synergen® on the *in sacco* degradation of feeds or on lactational responses when supplemented to cattle on high starch rations, would be worthwhile areas of investigation. Results from such studies would likely provide more robust evidence in favour of, or against, including Synergen® in cattle rations.

Chapter 4. The effects of a product of the solid-state fermentation of *Aspergillus niger* on *in sacco* degradation of feeds and rumen volatile fatty acid production in dairy cattle

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4.1 Abstract

This Research Paper addresses the hypothesis that the dietary inclusion of an *Aspergillus niger* fermentation product will alter the degradation kinetics and rumen fermentation patterns of feeds in dairy cattle. Fungal fermentation products often contain a suite of bioactive compounds and secondary metabolites which can influence the microbial environment in the rumen and act as digestibility enhancers. As the cattle sector is under increasing pressure to enhance its sustainability, the investigation of dietary interventions that could improve the efficiency of production is warranted. In a previous experiment, Synergen®, a product of the solid-state fermentation of *Aspergillus niger* (ANP) containing residual enzyme activities, significantly increased the *in vitro* digestibility of a grass silage-based dairy total mixed ration (TMR), suggesting that *in vivo* studies would be valuable. Hence the present study aimed to quantify the effects of this ANP on rumen fermentation measures in cattle. Using a 4 × 4 Latin square design, the effect of four doses of ANP (0, 5, 10, 15 g/d) in four cannulated Jersey heifers was measured on the *in sacco* degradation of dry matter (DM), organic matter, crude protein and neutral detergent fibre in steam-flaked barley, grass silage and a grass silage-based TMR formulated for dairy cattle. Treatments had no significant effect on the rate, or extent, of degradation of any component in any feed investigated. Rumen volatile fatty

acid concentrations and proportions, and rumen pH, were quantified at seven timepoints during each 48-h sampling period and were unaffected by treatment, as was the apparent total tract digestibility of DM. Under the conditions of this trial, ANP did not influence rumen fermentation kinetics; indicating that supplementing mature, non-lactating Jersey cattle with this fungal fermentation product is not an advantageous strategy to enhance feed digestibility.

Keywords: Ruminant, *in sacco* degradation, rumen fermentation, feed efficiency, fungal fermentation product

4.2 Introduction

The digestive efficiency of cattle is important for both economic and environmental agendas. The improvement of forage nutrient utilisation in cattle is directly linked with enhanced production and decreased greenhouse gas emissions intensity (GHG EI) (Arndt et al., 2022). The need to produce affordable food for a growing population, in an efficient and environmentally sustainable manner, remains a challenge faced by the entire global food system (Varzakas and Smaoui, 2024). The pressure of this challenge is acutely felt by the ruminant sector, which is frequently scrutinized for its contribution to GHG EI; especially for methane production from the ruminal microbial fermentation that enables ruminants to convert non-human edible feeds into highly nutritious human edible protein (Arndt et al., 2022). The inefficiencies of microbial fermentation in the rumen are well documented (Van Soest, 1994, Owens and Basalan, 2016); and a plethora of research has been conducted to investigate strategies to improve these inefficiencies.

Fungi can be a significant contributor to biomass in the rumen, estimates ranging from 8 % (Orpin, 1981) to 20 % of ruminal microbial dry matter (DM) (Rezaeian et al., 2004), and play a pivotal role in fibre digestion, as well as supplying the host animal with highly digestible amino acids (Hartinger and Zebeli, 2021). The abundance of rumen fungi is affected by the host's diet, with fungal populations, and activity, declining in highly digestible rations often fed to dairy cattle, compared to in animals fed rations high in lignified fibre, where fungi are

more abundant (Hartinger and Zebeli, 2021). Supplementing cattle rations with exogenous fungi, and their fermentation products, can offer a non-invasive method to enhance digestibility and positively manipulate the rumen microbiome towards more efficient fermentation pathways (Meale et al., 2014, Elghandour et al., 2023). The two dominant techniques for producing fungal fermentation products are solid-state fermentation (SSF) and submerged fermentation, the former occurring in the absence of free-flowing water and the latter in the presence of water. Both are methods of biotransformation, in which microbes are cultivated on a substrate to produce desirable end products, such as enzymes, organic acids, plant growth factors, alkaloids, biopolymers, aromas and pigments (Thomas et al., 2013).

Aspergillus spp. are commonly used in SSF in the food and feed industry (Thomas et al., 2013). Compared with the monogastric sector, less research has been conducted into the effects of *Aspergillus spp.* products in ruminants. However, studies have found their fermentation products to be efficacious at increasing the digestibility of cattle feeds *in vitro* (Kong et al., 2021, Tricarico and Dawson, 2005) and at enhancing digestibility and production responses *in vivo* (Caton et al., 1993, Tricarico et al., 2005, Sosa et al., 2022). Supplementing rations with *Aspergillus* spp. fermentation products does not always have an effect, with some studies finding limited or no response from treatments (Giraldo et al., 2007A, Sievert and Shaver, 1993). The findings of these studies demonstrate that sometimes, the inclusion of the fermentation products of *Aspergillus* spp. can increase the digestibility of feeds and favourably manipulate the products of fermentation. However, the conditions under which *Aspergillus* fermentation products are efficacious require defining; a common issue that hinders the industry utility of supplemental microbes and the products of their fermentation in ruminants (Meale et al., 2014, Amin and Mao, 2021).

Synergen® (Alltech Inc., Kentucky, US) is a commercially available product of the SSF of *Aspergillus niger* (ANP), containing residual enzyme activities. Previous research found that doses of 2 and 5 mg/g ANP significantly increased the *in vitro* gas production from a grass silage-based dairy total mixed ration (TMR),

suggesting that ANP could potentially enhance the digestibility of cattle rations (Yerby et al., 2025A). The goal of the present study was to investigate if ANP influenced *in vivo* rumen fermentation when fed to cattle, by measuring the effect of a range of doses of ANP on the *in sacco* degradation of dairy TMR, steam-flaked barley and grass silage, rumen volatile fatty acid (VFA) concentrations and proportions, rumen pH and the whole tract digestibility of DM.

4.3 Materials and methods

4.3.1 Study design

4.3.1.1 Animals and treatments

This trial followed a 4 × 4 Latin square design, investigating four doses of ANP (0, 5, 10, 15 g/d), in four cannulated, mature Jersey heifers, age 12 ± 2 years, weight 659 ± 37 kg. Doses of ANP were selected to represent range of inclusion rates around the 2 mg/g dose that previously increased *in vitro* TMR digestibility (Yerby et al., 2025A). A dose of 2 mg/g ANP *in vitro*, is approximately equivalent to feeding 10 g/d *in vivo*. Cattle were group-housed in a loose pen bedded with barley straw, with *ad libitum* access to a dry cow ration consisting of barley straw (750 g/kg) and grass silage (250 g/kg), on an as fed basis, which was topped up at 1500 h daily. Daily intake of the dry cow ration was not measured, but based upon mixer wagon top-ups, we estimate each heifer consumed approximately 20 kg/d, on an as fed basis. The nutritional composition of the ration is shown in Table 4-1. Each heifer was also fed 300 g/d concentrates comprising of 50 g steam-flaked barley and 250 g soaked sugar beet pulp, making the forage-to-concentrate ratio of the total ration approximately 99.4:0.6; cattle received trace elements and vitamins in a slow-release bolus (Rumbol, UK). ANP treatments were mixed with concentrates and each heifer was fed individually in a crush at 0800 h to ensure treatment consumption. Each trial period consisted of 21-d adaption to treatment, followed by 48-h of sampling,

with heifers randomly allocated treatments prior to the commencement of the trial.

Table 4-1. Nutritional composition of the dry cow ration, available ad libitum to four mature Jersey heifers, and the feeds suspended *in sacco*, in a 4 × 4 Latin square design trial, measuring the efficacy of four doses of Synergen on rumen fermentation kinetics.

	Grass silage	Dairy TMR[1]	Steam-flaked barley	Sugar beet pulp	Dry cow ration[2]
Dry matter (%)	19.7	35.6	92.8	90.0	70.3
Organic matter (%)	90.4	91.2	98.1	99.0	95.5
NDF (%)	49.9	35.3	15.1	38.2	74.2
ADF (%)	34	20.8	6	23.7	44.7
Crude protein (%)	19.8	16	11.2	8.0	8.1
Starch (%)	0.3	28.8	55.5	1.4	1.28
ME (MJ/kg)	10.3	10.3	12[3]	11.0	4.02

Abbreviations: TMR = total mixed ration; NDF = neutral detergent fibre; ADF = acid detergent fibre; ME = metabolizable energy. [1] *In sacco* dairy total mixed ration consisted of: 761 g/kg grass silage, 224 g/kg dairy concentrates (Advanced Robot 18 Dairy Nuts, Advanced Ruminant Nutrition), 15 g/kg molasses, on an as fed basis. [2] Dry cow ration consisted of: 750 g/kg barley straw, 250 g/kg grass silage, on an as fed basis. [3] 12 MJ (claimed by supplier – assay value out of range).

4.3.1.2 Parameters

The *in sacco* degradation of steam-flaked barley, grass silage and a grass silage-based dairy TMR comprising 761 g/kg grass silage, 224 g/kg dairy concentrates and 15 g/kg molasses, as fed, was measured following the recommendations in the method evaluation of Vanzant et al. (1998). The nutritional composition of feeds is shown in Table 4-1. Feeds were oven dried at 55 °C for 48-h and 5 g (± 5

%) of each feed was weighed into 672 pre-weighed ANKOM Forage bags (10 × 20 cm, 50 µm porosity). Feeds were dried to ensure homogeneity in DM across samples, as varying levels of moisture could impact the modelling of degradation kinetics, and to enable silage samples taken from the same clamp location to be used throughout the study; dried feeds were kept in airtight containers until required. During each sampling period, feed degradation was measured in duplicate at seven time points: 0, 3, 6, 9, 12, 24, 48-h, totalling 42 samples per rumen. Bags were soaked in 39 °C water for five minutes and then inserted into the ventral sac region of the rumen and anchored to the bung of the cannula with polyester string. At each collection timepoint, duplicate bags of each feed were removed from the rumen and placed into ice water to halt fermentation, prior to being washed in a washing machine on a 30-min cold cycle, and then oven dried at 55 °C for 48-h. Once dried, bags were re-weighed to calculate DM disappearance (Goering and Van Soest, 1970). The contents of duplicate bags were pooled and ground to pass through a 1 mm sieve (Retsch Cyclone, Germany) for neutral detergent fibre (NDF), crude protein (CP) and organic matter (OM) analyses. NDF concentrations of feed residuals were quantified following the procedure of Van Soest (1994), using an ANKOM^{200} Fibre Analyser (ANKOM Technology Corporation, NY. USA). The Kjeldahl method was used for estimation of nitrogen, with CP calculated as N × 6.25 (AOAC 2001.11) (Horwitz and Latimer, 2005). OM was determined by ashing feed residuals in a muffle furnace at 550 °C overnight (AOAC 923.03) (Helrich, 1990).

To quantify rumen pH and VFAs, ≈ 100 ml of digesta was collected from the ventral sac region of each rumen at timepoints: 0, 3, 6, 9, 12, 24, and 48-h, during each sampling period. The pH of digesta was measured using a calibrated probe (TRUEscience Smart Cap, UK) and then the digesta was strained through a double layer of cheesecloth. Duplicate 10 ml samples of strained rumen fluid were stored with 0.5 ml orthophosphoric acid in 15 ml Corning Centrifuge Tubes and frozen at − 20 °C until VFA analyses could be performed. The concentrations of total VFA, acetate, propionate, butyrate, valerate, isovalerate and isobutyrate in rumen fluid were subsequently measured with gas chromatography, using a 2-ethyl-butyric acid internal standard, following the

procedures described by Erwin et al. (1961). To measure apparent total tract digestibility of DM, duplicate rectal grab samples of faeces were obtained from each cow at the end of each sampling period. The acid insoluble ash (AIA) procedure of Van Keulen and Young (1977) was used to calculate DM digestibility. Only the AIA content of the dry cow ration was used in calculations, as only a small amount of concentrates were used as a mechanism to administer ANP, their contribution to total dietary AIA at an inclusion rate of approximately 0.6 %, was assumed to be negligible.

4.3.2 Modelling and statistical analysis of data

Degradation kinetics of DM, OM, NDF, and CP in feed residuals were estimated using the NOWAY programme (Harbron, 1994). NOWAY fits *in sacco* degradability values to the exponential decay equation described by Ørskov and McDonald (1979):

$$Y = a + b(1 - e^{-ct})$$

Y = the degradation of the substrate at rumen incubation time *t*

a = the immediately soluble fraction + small particle insoluble fraction of the substrate (%) at *t* = 0

b = the insoluble, slowly degradable fraction of the substrate (%)

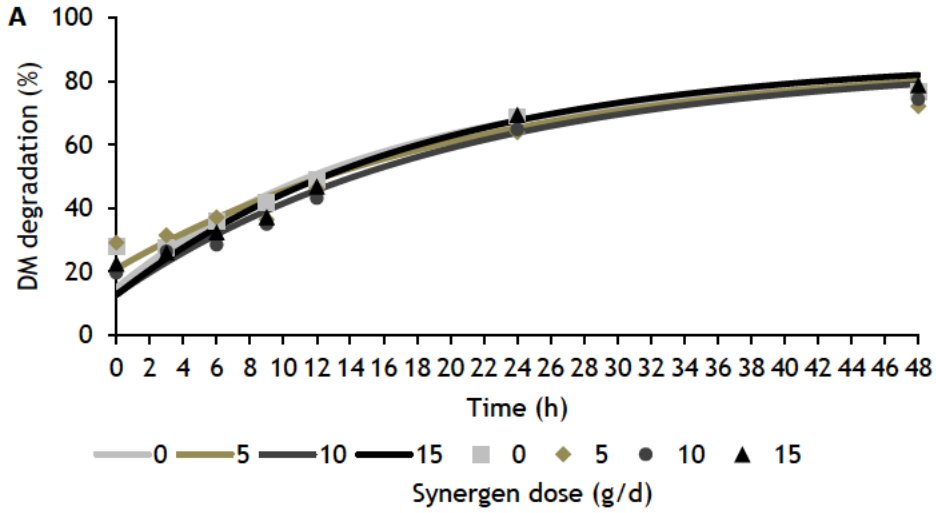
a + *b* = the potential degradability of the substrate when *t* is not limited

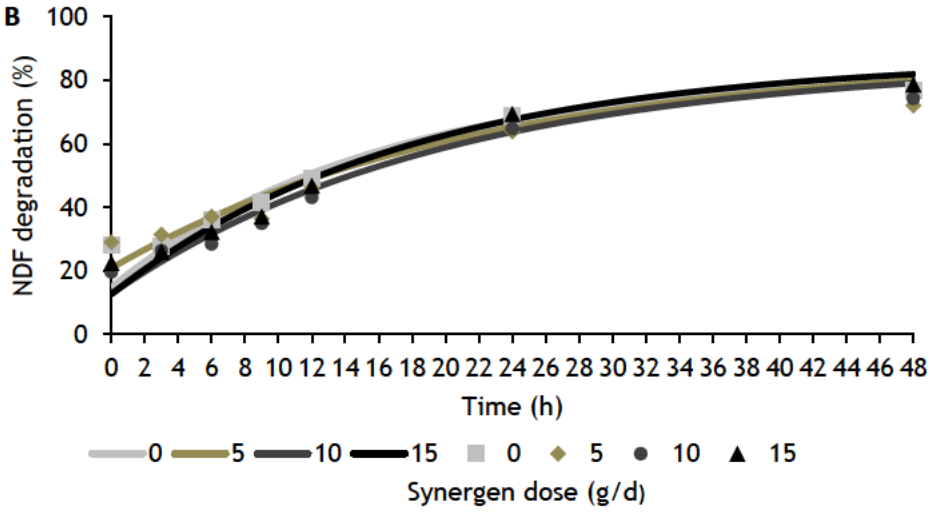
c = the fractional rate at which *b* is degraded (%/h)

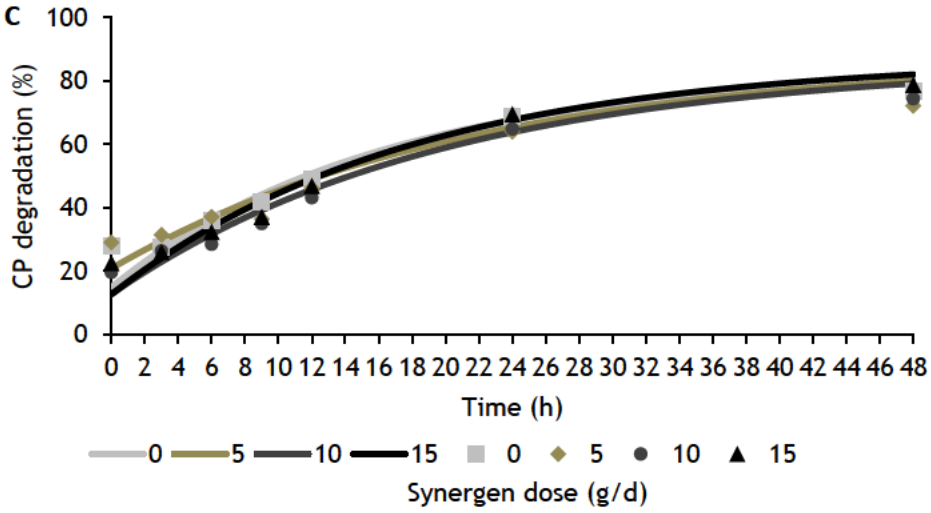
Datasets were subjected to a Shapiro Wilks normality test and differences between *a, b,* and *c* parameters were calculated with one-way ANOVA. Differences in rumen pH, total tract digestibility of DM, and rumen VFA proportions and concentrations were calculated with repeated measures ANOVA. All statistical analyses were performed in R (R Core Team, 2024) and significance was declared at $p < 0.05$.

4.4 Results

Fig. 4-1, Fig. 4-2 and Fig. 4-3 show the degradation curves of DM (**A**), NDF (**B**), CP (**C**), and OM (**D**) components of 5 g grass silage, grass silage-based dairy TMR and steam-flaked barley, respectively. Plotted lines are the fitted degradation curves calculated using the NOWAY programme, individual points are the mean degradation values of each component at each bag-withdrawal timepoint. The mean *a*, *b* and *c* values estimated by the NOWAY programme for the degradation of DM, OM, NDF and CP in each feed over 48-h are presented in Table 4-2. There were no significant differences between any treatments for any degradation parameter. The mean apparent total tract digestibility of DM from treatments of 0, 5, 10 and 15 g ANP was 83.6, 79.8, 82.1 and 80.3 %, respectively (pooled SE = 1.08). There were no significant differences in apparent digestibility from treatment (p = 0.639), experimental period (p = 0.948) and no treatment × period interaction (p = 0.721).




B
NDF degradation (%)
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (h)
0 5 10 15 0 5 10 15
Synergen dose (g/d)


C
CP degradation (%)
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (h)
0 5 10 15 0 5 10 15
Synergen dose (g/d)

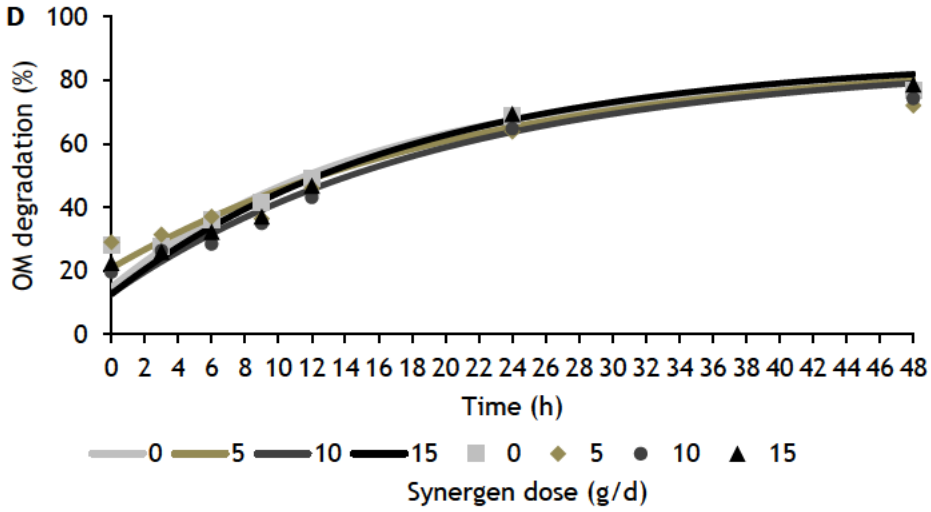
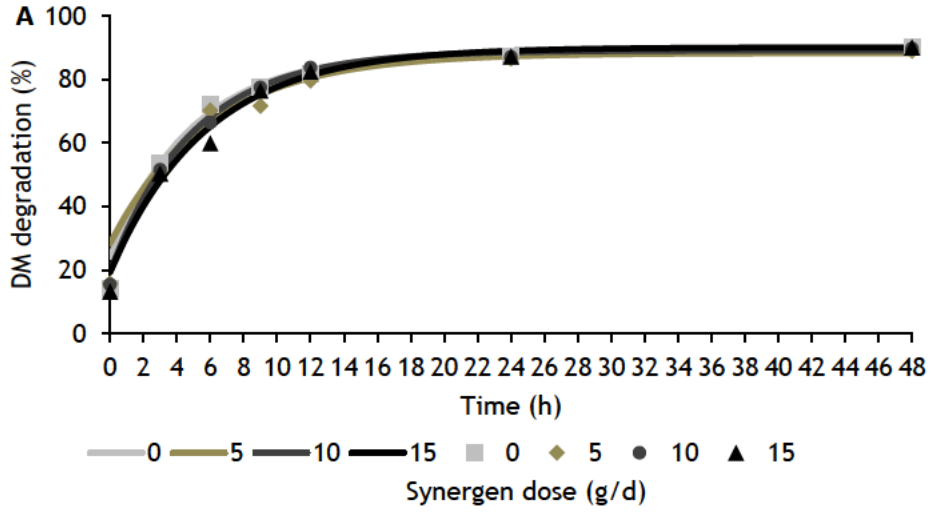
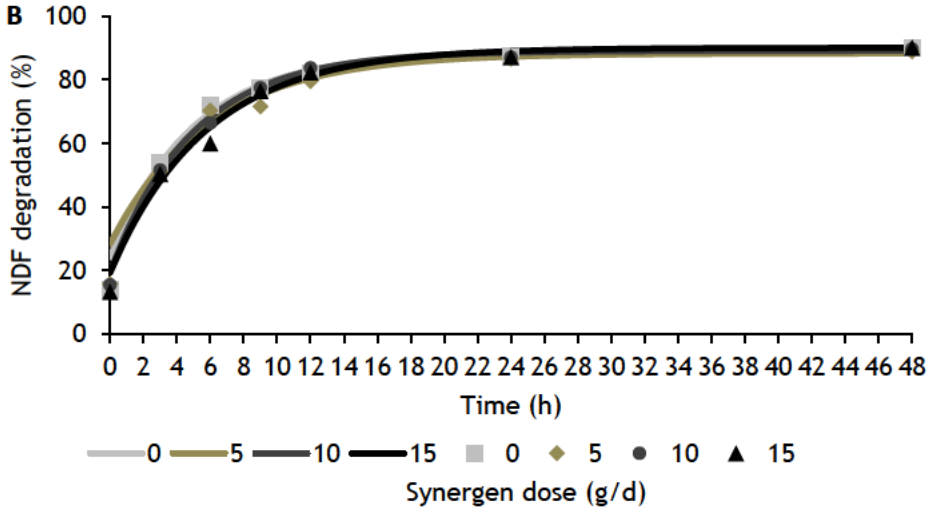


Figure 4-1. Degradation curves of the dry matter (A), neutral detergent fibre (B), crude protein (C), and organic matter (D) components of 5 g grass silage incubated in the rumens of four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. Plotted lines display the degradation curves calculated using the NOWAY programme (Harbron, 1994). NOWAY estimates the degradation kinetics of feed, using the exponential decay equation described by Ørskov and McDonald (1979). Individual scatter plot points display the mean degradation values of each component, quantified at seven bag withdrawal timepoints (0, 3, 6, 9, 12, 24, and 48-h).


A
DM degradation (%)
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (h)
0 5 10 15 0 5 10 15
Synergen dose (g/d)


B
NDF degradation (%)
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (h)
0 5 10 15 0 5 10 15
Synergen dose (g/d)

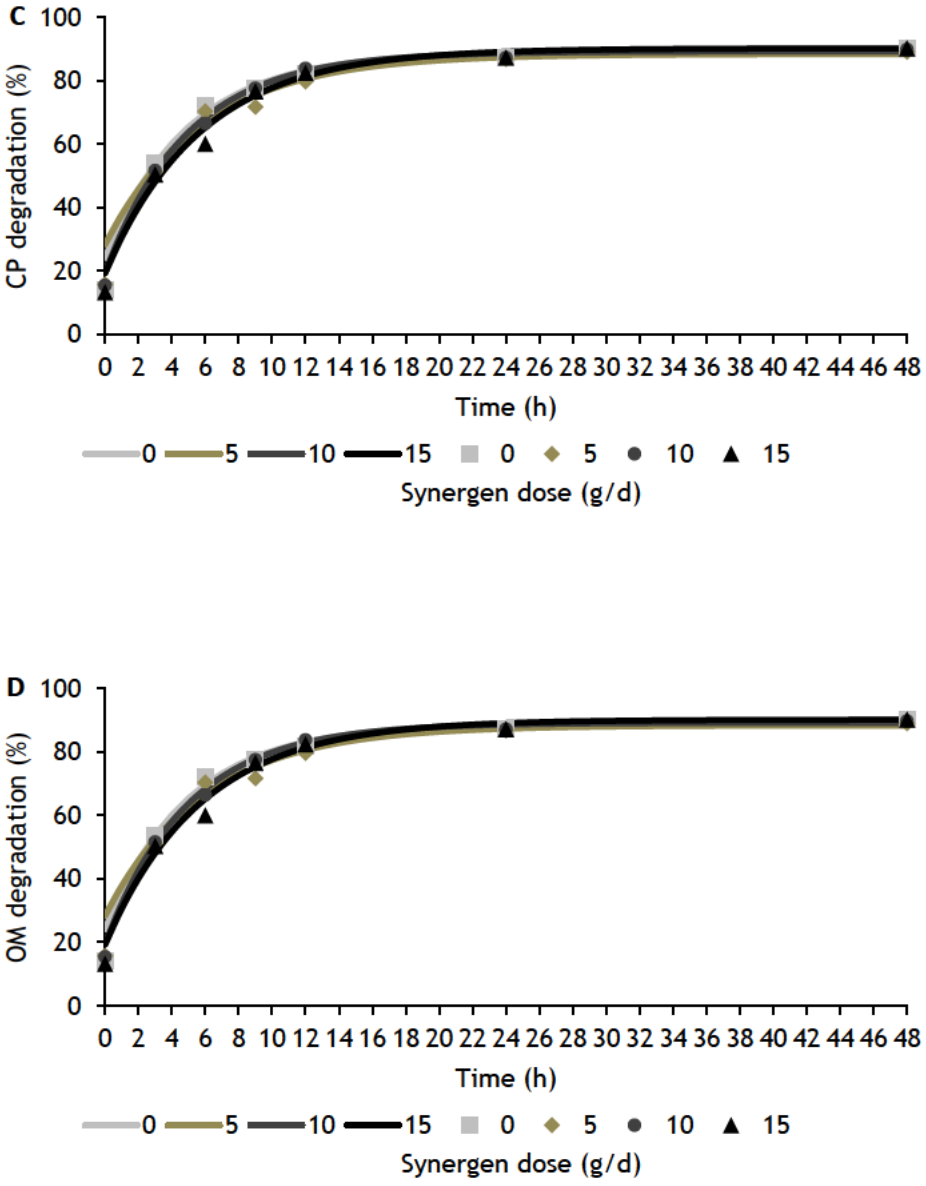
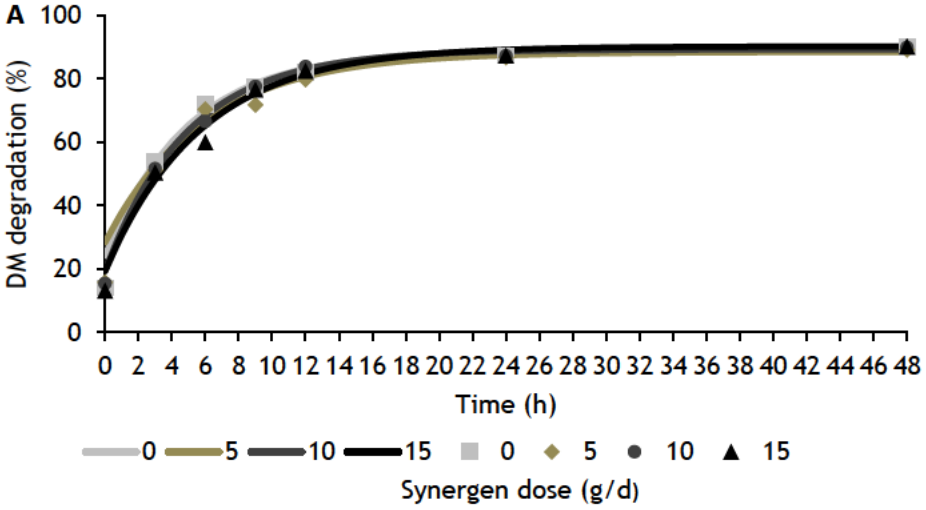
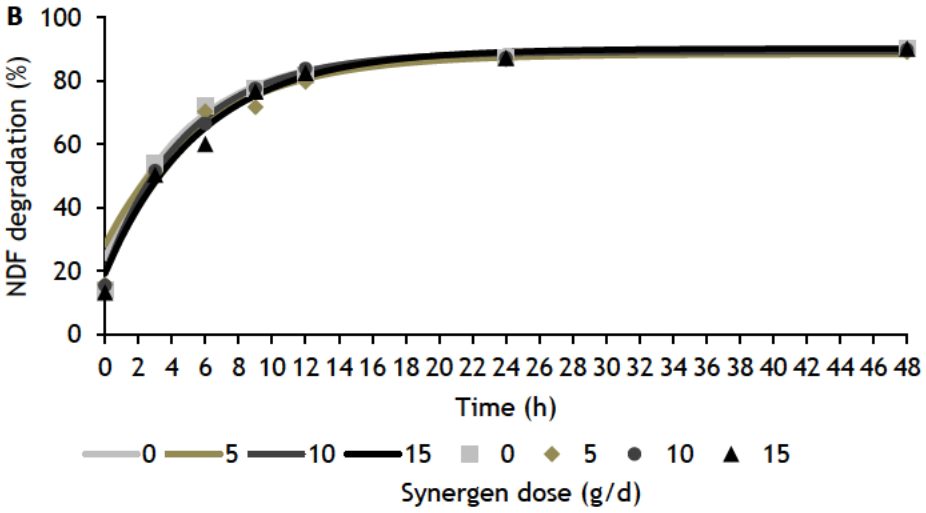


Figure 4-2. Degradation curves of the dry matter (A), neutral detergent fibre (B), crude protein (C), and organic matter (D) components of 5 g grass silage-based dairy TMR incubated in the rumens of four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d) in a 4 × 4 Latin square design digestibility trial. Plotted lines display the degradation curves calculated using the NOWAY programme (Harbron, 1994). NOWAY estimates the degradation kinetics of feed, using the exponential decay equation described by Ørskov and McDonald (1979). Individual scatter plot points display the mean degradation values of each component, quantified at seven bag withdrawal timepoints (0, 3, 6, 9, 12, 24, and 48-h).


A
DM degradation (%)
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (h)
0 5 10 15 0 5 10 15
Synergen dose (g/d)


B
NDF degradation (%)
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (h)
0 5 10 15 0 5 10 15
Synergen dose (g/d)

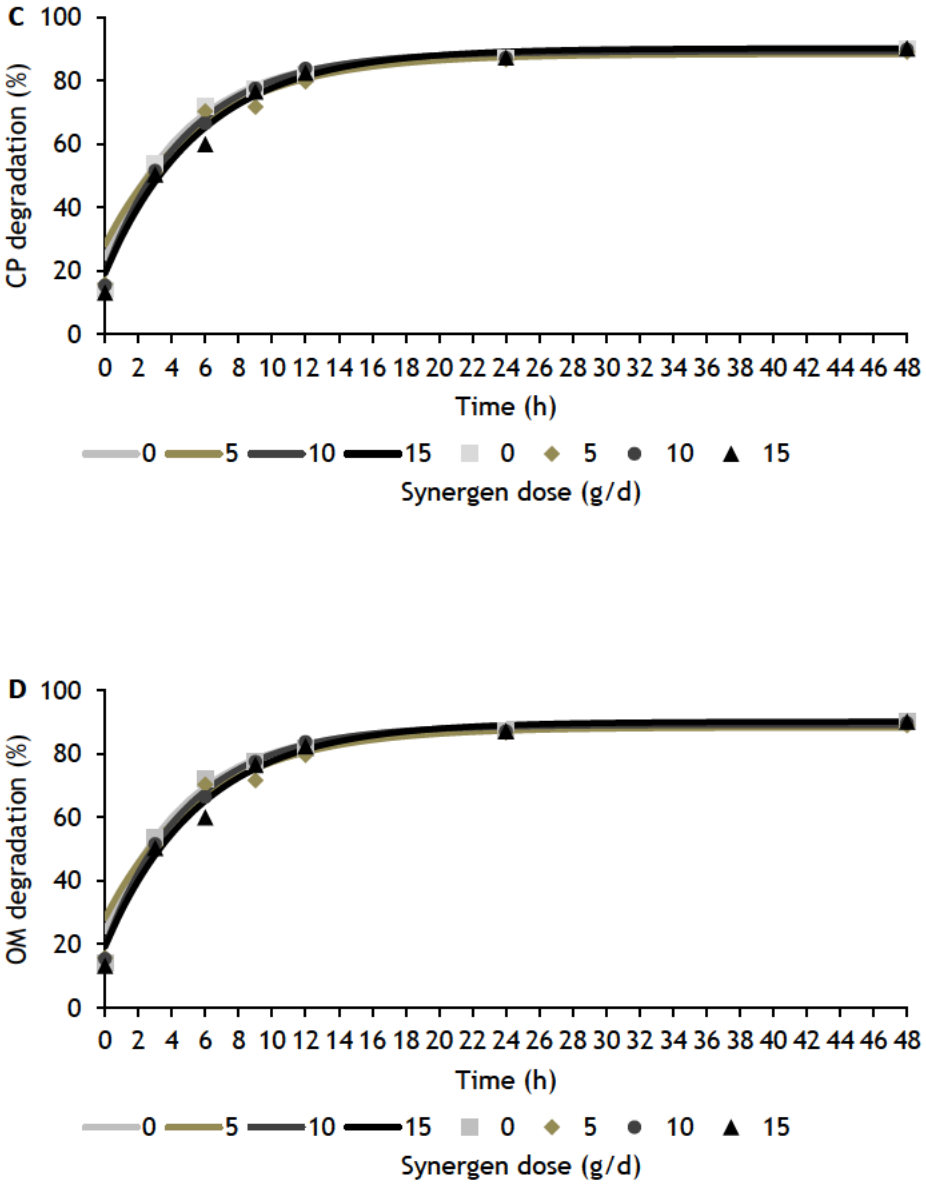


Figure 4-3. Degradation curves of the dry matter (A), neutral detergent fibre (B), crude protein (C), and organic matter (D) components of 5 g steam flaked barley incubated in the rumens of four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. Plotted lines display the degradation curves calculated using the NOWAY programme (Harbron, 1994). NOWAY estimates the degradation kinetics of feed, using the exponential decay equation described by Ørskov and McDonald (1979). Individual scatter plot points display the mean degradation values of each component, quantified at seven bag withdrawal timepoints (0, 3, 6, 9, 12, 24, and 48-h).

Table 4-2. Exponential decay parameters estimated by the NOWAY programme (Harbron, 1994) for the degradation of dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), and crude protein (CP) components of 5 g grass silage, grass silage-based dairy TMR, and steam-flaked barley.

Substrate	Nutrient	Parameter	Synergen dose (g/d)				Pooled SE	*P*- value
			0	5	10	15		
Grass silage	DM	a	20.0	25.2	18.3	17.9	2.025	0.6069
		b	62.9	62.6	67.9	69.2	2.263	0.6872
		c	0.064	0.047	0.05	0.055	0.006	0.7349
	OM	a	14.9	20.8	12.7	12.6	2.135	0.5281
		b	67.5	66.5	73.0	74.4	2.444	0.6358
		c	0.063	0.046	0.05	0.056	0.005	0.7299
	NDF	b	83.5	73.2	89.4	92.1	3.670	0.2900
		c	0.063	0.047	0.048	0.057	0.004	0.5414
	CP	a	50.5	55.1	52.0	54.0	1.896	0.8650
		b	36.0	32.2	40.9	36.7	1.948	0.5883
		c	0.1	0.081	0.059	0.058	0.011	0.5374
Dairy TMR	DM	a	28.8	28.0	28.8	29.4	1.966	0.9969
		b	55.7	57.4	56.2	56.2	2.100	0.9948
		c	0.086	0.077	0.081	0.076	0.005	0.9072
	OM	a	25.9	25.3	25.9	26.5	2.041	0.998
		b	58.4	60.0	59.1	59.0	2.198	0.9968
		c	0.086	0.077	0.08	0.076	0.005	0.9032

	NDF	b	74.0	73.6	79.7	72.5	3.098	0.8740
		c	0.065	0.054	0.075	0.057	0.006	0.5991
	CP	a	56.8	54.4	56.3	58.4	2.010	0.9350
		b	31.8	36.7	34.9	32.7	2.296	0.8992
		c	0.096	0.073	0.078	0.069	0.009	0.7509
Barley	DM	a	24.4	28.5	19.7	19.7	3.643	0.8298
		b	64.3	59.7	69.3	70.3	3.688	0.7656
		c	0.201	0.168	0.2	0.173	0.013	0.7374
	OM	a	24.2	28.4	19.5	19.3	3.704	0.8309
		b	64.6	60.0	69.6	70.8	3.749	0.7634
		c	0.203	0.170	0.201	0.174	0.013	0.7499
	NDF	b	44.7	59.0	53.9	49.5	4.324	0.7211
		c	0.058	0.052	0.113	0.133	0.019	0.3667
	CP	a	12.3	14.0	0.8	9.0	3.179	0.5082
		b	87.3	79.3	100	97.8	4.795	0.4090
		c	0.153	0.106	0.172	0.134	0.014	0.4446

Abbreviations: a = the immediately soluble fraction of the substrate (%) at timepoint 0; b =the insoluble, slowly degradable fraction of the substrate (%); c = the fractional rate at which b is degraded. Feeds were incubated in the rumens of four mature Jersey heifers, fed four doses of Synergen® (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. Degradation was measured at seven time points (0, 3, 6, 9, 12, 24, and 48-h) during 48-h sampling periods.

Figure 4-4 shows the mean pH of rumen digesta collected from the fistulated cattle at seven timepoints during each 48-h sampling period. There was no effect of treatment on rumen pH (p = 0.946), nor was there a treatment × time

interaction (p = 0.821). The effect of time on rumen pH was significant (p < 0.001). Mean concentrations and proportions of VFAs measured in rumen digesta are presented in Table 4-3. There was no significant effect of treatment, or a significant treatment × time interaction for any absolute or proportional VFA values. For all VFA parameters, the effect of time was significant (p < 0.001).

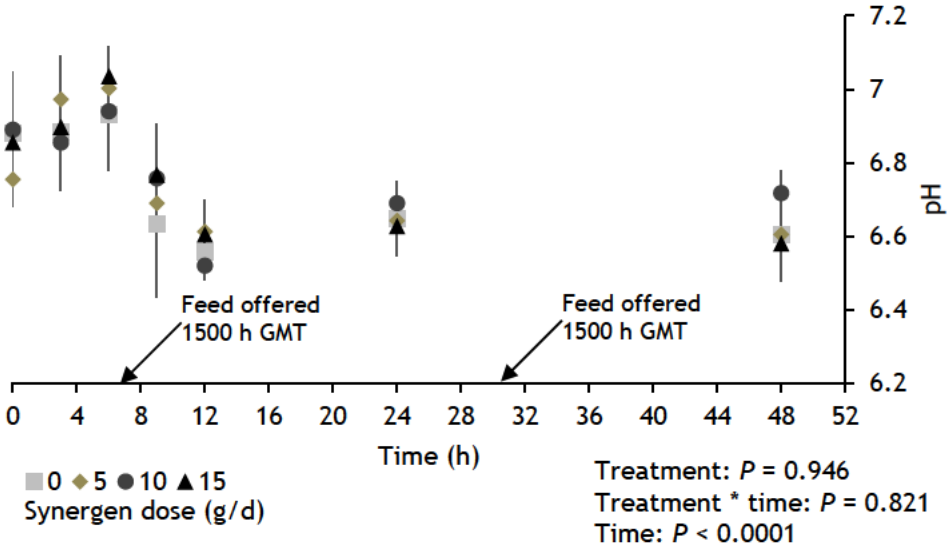


Figure 4-4.Rumen pH in four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. pH of digesta was measured at seven time points during 48-h sampling periods. Error bars display SEM, and *P* values were calculated using Repeated Measures Two-way ANOVA in R.

Table 4-3. Mean rumen volatile fatty acid concentrations and proportions in four mature Jersey heifers, fed four doses of Synergen (0, 5, 10, 15 g/d), in a 4 × 4 Latin square digestibility trial. Digesta samples were collected from the ventral sac region of each rumen at seven time points during 48-h sampling periods.

Parameter	Synergen dose (g/d)				Pooled SE	Treatment *P*-value	Treatment * Time *P*-value
	0	5	10	15			
tVFA (mM)	116	121	120	116	6.198	0.980	0.698
Acetate (mM)	83.1	87.7	87.4	82.7	4.950	0.956	0.633
Propionate(mM)	20.7	21.1	20.4	21.1	1.116	0.993	0.909
Butyrate (mM)	10.0	10.3	10.2	10.0	0.324	0.977	0.844
Valerate (mM)	0.629	0.530	0.552	0.669	0.058	0.797	0.985
Isovalerate (mM)	0.745	0.776	0.649	0.818	0.066	0.799	0.959
Isobutyrate (mM)	0.581	0.517	0.478	0.665	0.046	0.380	0.769
Acetate: propionate	4.08	4.17	4.27	3.97	0.085	0.538	0.633
Acetate (% tVFA)	70.4	70.7	71.0	70.2	0.491	0.890	0.751
Propionate (% tVFA)	17.9	17.5	17.4	18.2	0.288	0.745	0.957
Butyrate (% tVFA)	9.43	9.46	9.54	9.33	0.175	0.966	0.436
Valerate (% tVFA)	0.693	0.643	0.653	0.694	0.052	0.969	0.949
Isovalerate (% tVFA)	0.857	0.925	0.775	0.888	0.065	0.799	0.900
Isobutyrate (% tVFA)	0.706	0.707	0.626	0.732	0.051	0.814	0.675

The effect of time was significant ($P < 0.001$) for all parameters.

4.5 Discussion

In the present study, none of the doses of ANP influenced the degradation of feeds *in sacco*, the apparent total tract digestibility of DM, or the VFA production from rumen fermentation. When we trialled this ANP *in vitro,* significant increases in gas production were measured, suggesting increased digestibility (Yerby et al., 2025A). The ANP doses and TMR used in the *in vitro* study were similar to the treatment doses and TMR used in the present study, and as such, we anticipated the ANP to influence digestibility *in vivo*. Although *in vitro* gas production is a valuable tool for highly controlled pre-screening of feed materials and additives prior to animal testing, *in vitro* effects do not guarantee efficacy in animals, as demonstrated by these results. The rumen is a complex organ containing a dynamic population of microbes. As such, many factors, including dose, production method, animal age and productivity, environment, pH stability, and feed specificity, can influence the efficacy of a microbial fermentation product in the rumen (Beauchemin et al., 2004). To the authors' knowledge, no *in vivo* studies have been published on the effects of fermentation products solely from *A. niger* in mature cattle. However, there have been inconsistent responses in studies measuring the effects of products from a closely related species: *Aspergillus oryzae*, and combined *A. oryzae* and *A. niger* co-cultivation products, on rumen fermentation kinetics and cattle productivity.

Yoon and Stern (1996) supplemented four lactating Holstein cows with 3 g/d *A. oryzae* culture in a 4 × 4 Latin square design, as well as a *S. cerevisiae* treatment and a combination of *S. cerevisiae* and *A. oryzae*. Cows treated with *A. oryzae* culture had a significant increase in ruminal cellulolytic and proteolytic bacterial counts. However, similar to our study, treatment had no effect on ruminal digestibility, rumen pH, whole tract digestibility, or VFA concentrations and proportions. Although a combination of *A. oryzae* and *S. cerevisiae* did decrease ruminal isoacid concentrations. Increased populations of cellulolytic and proteolytic bacteria should influence rumen fermentation (Newbold et al.,

1992). However, no significant effect on fermentation parameters was measured by Yoon and Stern (1996). Conversely, Sosa et al. (2022) trialled four doses of *A. oryzae* culture (0, 1, 2, 3 g/d), in four non-lactating Holstein × Zebu cows, in a 4 × 4 Latin square design trial, and found a significant increase ($p < 0.0001$) in ruminal cellulolytic bacteria and fungi from all treatment doses, paired with a significant increase in DMI. Sosa et al. (2022) also realised an increase in total VFA production from the 2 g/d dose of *A. oryzae* culture, which is likely related to the increase in cellulolytic microbes. Notably, the same *A. oryzae* dose (3 g/d) as used in the study of Yoon and Stern (1996), did not significantly influence VFA production or proportions. This highlights the impact that factors such as inclusion rate can have on the efficacy of *Aspergillus spp.* products. The cattle in the study of Sosa et al. (2022) were fed a highly digestible ration, containing 664 g/kg DM corn silage and 300 g/kg DM soybean, in comparison to the ration offered in our study which was straw based and high in undigestible fibre. The rumens of cattle fed high fibre diets are favourable environments for anaerobic fungi to proliferate in, and these fungi can enhance the digestion of lignified fibre with penetrative hyphae (Hartinger and Zebeli, 2021). Sosa et al. (2022) measured significant increases in rumen fungi from 3.35 log ufc/mL in control cattle, to 5.34 log ufc/mL in cattle fed 2 g/d *A. oryzae* extract, which likely contributed to the improvements in performance measured in their study. Although fungi populations were not measured in the present study, it is plausible that the high levels of endogenous fungal activity resulting from basal diet offered, may have caused the lack of treatment effects in this study.

Martins et al. (2022) measured the effect of an enzyme extract from *A. niger* and *A. oryzae* on the total tract digestibility and lactational productivity of primiparous and multiparous Holstein cows ($n = 24$ per control and treatment groups), offered a TMR containing 37.5 % cereals, 43 % corn silage, 13.2 % alfalfa haylage and 4 % straw hay mix, as fed. The extract contained amylase, hemicellulase, cellulase, ß-glucanase, and pectinase activities, and was included at a dose rate of 113.4 g/d. As with our study, total tract digestibility was unaffected by treatment. However, treatment increased milk yield and energy-corrected milk in primiparous cows, but not multiparous cows. Concentrations of

milk protein and lactose were increased from treatment regardless of parity but yields of milk protein and lactose were only increased in primiparous cows. Somatic cell count was significantly reduced by treatment irrespective of parity. There was no effect of treatment on body weight, milk urea nitrogen, or total milk solids. This study indicates that lactational performance of cattle can be enhanced from *Aspergillus spp.* products, without a corresponding significant increase in whole tract nutrient digestibility. Notably, the parity × treatment interactions for yields of milk, protein, and lactose, highlight the influence that age can have on the efficacy of microbial fermentation products. In their first lactation, many cows have not reached their mature size, thus, DMI can be limited by GI tract capacity (Van Soest, 1994). Enhancing the hydrolytic capacity of the rumen with *Aspergillus spp.* products could increase rumen outflow rate and consequently DMI, although this was not measured by Martins et al. (2022). Increased DMI could enable increased VFA production and could explain beneficial lactation responses. The rumen microbiome was not examined in this study, but the ration cattle were fed would likely provide a less favourable environment for endogenous fungi compared to the ration offered in our study, and as such, the addition of this *Aspergillus* extract may have had a greater capacity to enhance rumen fermentation.

Sun et al. (2017) measured the effect of 5 g/d *A. oryzae* culture in multiparous Chinese Holstein cows (*n* = 24 per control and treatment groups). Similarly to the multiparous cows in the study of Martins et al. (2022), treatment had no effect on the milk yield, fat or protein; although an increase in the percentage of lactose in milk was realised. Conversely to our study, cows treated with *A. oryzae* culture had significantly greater production of VFAs and increased microbial protein production; coupled with a significantly higher *Ruminococcus flavefaciens* population and increased *carboxymethylcellulase* activity in their rumens. Suggesting that *A. oryzae* influences the rumen microbial community and increases its hydrolytic capacity. In our study, the cattle treated with ANP were not lactating, and considerably older (12 ± 2 years) and heavier (659 ± 37 kg) than most Jersey cows in commercial herds, as well as being offered a high fibre diet unsuitable for lactating animals. Their metabolic demands would be

considerably lower than lactating and/or pregnant cows (Ferrell and Jenkins, 1984). Although cannulating cattle allows for access into the rumen environment, which can provide valuable data on rumen fermentation parameters which would otherwise be difficult to collect, the physiological and dietary differences between the cattle available for use in this study and cattle in dairy herds is a limitation of this study. The addition of small quantities of fungal fermentation product, without penetrative capacity due to loss of the hyphal function following SSF, into an environment which could already have significant fungal activities, likely constrained the potential efficacy of this ANP.

4.6 Conclusion

In the present study, supplementing mature, non-lactating Jersey cattle with ANP had no effect on the *in sacco* degradation of grass silage, grass silage-based dairy TMR, or steam-flaked barley. Treatments did not alter the pH of the rumen, the production of VFAs, or the total tract digestibility of DM. These results indicate that under the conditions of this trial, supplemental ANP does not influence digestibility or the end products of rumen fermentation. Further research is required to determine if age, breed, diet and lactation influence the efficacy of ANP in cattle.

4.7 Annex A

Additionally to the analysis presented in the body of Chapter 4, the kinetic parameters of feed fractions estimated using the NOWAY programme (Harbron, 1994) were used to estimate effective degradabilities. These estimates are presented in Table 4-4 and were calculated using the equation of Ørskov and McDonald (1979), using a value of 0.05 h^{-1} for rumen outflow (k):

$$Effective\ degradability = a + (b\ \times c)/(c + k)$$

a = the immediately soluble fraction + small particle insoluble fraction of the substrate (%)

b = the insoluble, slowly degradable fraction of the substrate (%)

c = the fractional rate at which *b* is degraded (h^{-1})

k = rumen outflow date (h^{-1})

Differences in estimates of effective degradabilities between ANP doses were calculated using one-way ANOVA with significance declared at $p < 0.05$. Aligned with the data presented in the body of Chapter 4, there were no differences between any ANP doses for the effective degradability of any feed fraction assessed.

Table 4-4 Estimates of effective degradabilities of dry matter, organic matter, neutral detergent fibre, and crude protein components of 5 g grass silage, grass silage-based dairy TMR, and steam-flaked barley incubated in the rumens of fistulated Jersey cattle.

		Effective degradability (%) at ANP dose (g/d):					
Substrate	Nutrient	0	5	10	15	Pooled SE	*P* value
Grass silage	DM	54.5	52.3	50.4	53.2	1.86	0.905
	OM	51.8	49.6	47.4	50.5	2.01	0.909
	NDF	46.1	35.2	41.6	45.6	1.82	0.109
	CP	72.8	74.5	71.2	72.6	1.54	0.936
Dairy TMR	DM	63.8	62.4	63.2	62.8	0.85	0.956
	OM	62.5	61.2	61.9	61.6	0.87	0.968

	NDF	40.9	37.0	47.2	38.3	2.79	0.622
	CP	77.7	74.9	75.8	76.4	0.78	0.681
Barley	DM	75.9	74.3	75.0	73.8	0.65	0.705
	OM	76.1	74.4	75.1	73.9	0.65	0.706
	NDF	17.5	22.3	35.0	35.4	3.83	0.234
	CP	71.1	66.4	68.8	66.8	1.24	0.566

Abbreviations: ANP = *Aspergillus niger* fermentation product; DM = dry matter; OM = organic matter; NDF = neutral detergent fibre; CP = crude protein; TMR = total mixed ration; SE = standard error.

Feeds were incubated in the rumens of four mature Jersey heifers, fed four doses of ANP (0, 5, 10, 15 g/d), in a 4 × 4 Latin square design digestibility trial. Degradation was measured at seven time points (0, 3, 6, 9, 12, 24, and 48-h) during 48-h sampling periods.

Chapter 5. Systematic review: Efficacy of exogenous enzymes for dairy calves

Bridge to Chapter 5

The lack of treatment effects measured when ANP was trialled in mature cattle suggested that the inclusion of the product in dairy rations may not be an effective dietary intervention. As discussed in Chapters 2 - 4, there are many factors which can limit the efficacy of fungal fermentation products in cattle; in our *in sacco* study, the age, weight and diet of the fistulated cattle available for use may have limited the efficacy of ANP. As such, it was decided that this project should change its focus from mature dairy cattle to calves. There is evidence demonstrating that ANP is effective at increasing the digestibility of feeds and improving animal performance in monogastric animals (Perić and Spring, 2013, Diógenes et al., 2018) and the product is used commercially in aquaculture and poultry enterprises. Given that calves are born with digestive systems akin to monogastrics, we hypothesized that ANP could confer beneficial effects to calves when fed in the pre-ruminant and transition stages. To aid in the design of a calf study, a systematic review of current literature exploring enzymatic product use in calves was conducted and is presented in Chapter 5 of this thesis.

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5.1 Abstract

The aim of this review is to quantify the strength of evidence for the efficacy of exogenous enzymes (EE) in dairy calf rearing, by systematically identifying, consolidating, and discussing existing research on the subject. This review identified 17 articles that measured the effect of EE alone or in combination with another treatment on indicator variables for calf performance, behaviour, health or environmental output; 15 studies realised a positive effect of enzyme

supplementation on at least one variable, and one study realised a negative effect of treatment on body parameters. Inconsistent results were noted for effects of EE on growth and feed efficiency. Studies that combined EE with another treatment did not tend to find performance improvements compared to standalone treatments. However, several studies were underpowered which could have limited their ability to detect effects on primary response variables. All papers that measured digestibility found an increase in fibre digestibility from enzyme treatment; however, this did not always result in improved feed efficiency or growth. Reductions in non-nutritive oral behaviour, increased resting time and rumination, were observed in studies measuring calf behaviour, as were enhanced rumen development and reduced cost of calf rearing; suggests that EE could enhance calf health and welfare, whilst having additional advantages for rearing economics. Future research into the efficacy of EE in dairy calves is worth pursuing. However, a targeted and evidence-based approach to experimental design is required, with due consideration given to enzyme actions and interactions, as well as robust power analysis for sample size.

Key words: Dairy calves; exogenous enzyme; nutrition; calf performance; Systematic review.

5.2 Introduction

5.2.1 Rationale

Nutritional interventions to optimise calf growth and wellbeing in the neonatal and transition periods can have a significant impact on their performance and productivity when they join the milking herd (Davis and Drackley, 1998). Limited research has been conducted on the effects of EE in dairy calves. Supplemental EE could enhance rumen development and the propagation of the rumen microbial ecosystem that enables calves to derive nutrients from solid feeds, which is vital for calf wellbeing and performance (Davis and Drackley, 1998). The incorporation of EE, typically of bacterial or fungal origin, into the diets of mature dairy cattle has been widely researched and found to often be an advantageous strategy to increase the hydrolytic capacity of the rumen and manipulate the rumen microbiome (Beauchemin et al., 2003). Sometimes this leads to improved feed efficiency and favourable products of fermentation, which consequently produces favourable outcomes for cattle health, production and environmental sustainability (Beauchemin et al., 2003). Hundreds of studies have been published on the effects of EE in adult dairy cattle which indicate that the use of EE for ruminant production is usually beneficial for feed efficiency and productivity (Beauchemin et al., 2003, Meale et al., 2014, Sujani and Seresinhe, 2015, Arriola et al., 2017, Tirado-González et al., 2021). However, inconsistencies in responses to treatment, which can be attributed to factors such as: enzyme source, cultivation method, application technique, dietary interactions, varying dose rates, retention time, pH stability and ionic strength, have meant that the use of EE in the ruminant sector, although under intense research, has not yet become a commonplace feeding strategy (Wang and McAllister, 2002, Sujani and Seresinhe, 2015). Undertaking a Systematic review of research examining the effects of EE supplementation in dairy calves will allow for the identification of knowledge gaps and quantification of the strength of evidence in favour of their use.

5.2.2 Calf rearing in the dairy industry

Early weaning of dairy calves means that the transition from pre-ruminant to ruminant occurs at a young age, where the need for dairy calves to derive nutrients from solid feeds just weeks after parturition is much greater than their beef suckler counterparts (Drackley, 2008). Artificial rearing of dairy calves is standard practice for modern dairy systems. For a herd being milked through a parlour or robot, rearing calves artificially is undoubtedly more economical and practical than natural suckling, a practice where calves in beef herds or the wild could be left to nurse for up to 14 months (Reinhardt and Reinhardt, 1981). However, being reared away from its dam is challenging for a calf, which is reflected in relatively higher levels of morbidity and mortality in artificial rearing systems (Windeyer et al., 2014). Two of the main distinctions between natural and artificial rearing are the length of time the calf is fed milk or milk replacer, and the quantity of milk provided. In European dairy systems, calves are typically weaned from 6 to 12 weeks of age and are often fed a restricted quantity of milk, approximately 10 % of their body weight (BW) (Drackley, 2008). In contrast, calves in cow-calf herds are usually weaned much later, from 6 – 14 months of age, and consume approximately 20% of their BW in milk until natural weaning when the dam begins to produce less milk and the solid feed intake of the calf increases to compensate (Enríquez et al., 2011). Limiting the milk intake and weaning age of dairy calves is economically desirable, with solid feeds costing considerably less than whole milk and milk replacer. However, early weaning and restricted milk intake can have negative implications for calf performance; especially if their rumen is insufficiently developed to derive the necessary nutrients from solid feed for maintenance and growth at the point of weaning (Khan et al., 2016). Hence strategies that use EE to enhance feed digestion and rumen development could improve calf productivity and welfare in modern dairy systems.

5.2.3 Neonatal calves

Calves are born with a four-compartment stomach, however, the rumen, reticulum and omasum are underdeveloped and the abomasum makes up approximately 70 % of the total digestive tract (Huber, 1969). When calves suckle, the reticular groove delivers milk directly from the oesophagus to the omasum, bypassing the rumen and reticulum, enabling milk to be digested most efficiently in the abomasum by autoenzymatic digestion, predominantly by rennin and lactase. The diversity and abundance of enzymes found in the abomasum increase as the calf matures and its diet changes, these enzymes include pepsin, lipases and amylases (Huber, 1969). When calves are in the pre-ruminant stage, their digestive physiology is analogous to monogastrics, relying on autoenzymatic digestion rather than the alloenzymatic digestion that occurs in a developed rumen. In contrast to the ruminant nutrition sector, where widescale industry uptake of EE as a feeding strategy has been limited by inconsistent responses to enzyme supplementation, EE have been successfully and widely used in monogastrics. This began with Hervey (1925), who realised an increase in growth rates and final body weights of Leghorn chicks from the dietary inclusion of fungal enzymatic material. Their research acted as a springboard for innumerable studies finding beneficial outcomes for EE use in pigs, poultry and aquaculture, which has subsequently led to the widescale use of EE in the monogastric sector (Bedford and Schulze, 1998). When considering why monogastric responses to EE are typically beneficial, and ruminant responses are more inconsistent, it is reasonable to postulate that a major factor is because monogastric animals are autoenzymatic digesters (Bedford, 2000). They have no access to the battery of endogenous enzymes provided by the rumen microbiome, therefore, increasing the hydrolytic capacity of their digestive system using EE has a much greater impact on nutrient utilization and consequently animal performance (Asmare, 2014). Given the physiological similarities between pre-ruminant and monogastric digestive processes, EE supplementation in calves may have greater, and perhaps more consistent, effects than those observed when supplementing mature cattle.

5.2.4 Transitioning from pre-ruminant to ruminant

In calves, the transition from pre-ruminant to ruminant occurs from 4 to 12 weeks of age, when microbial fermentation is initiated in the rumen by the consumption of water and solid feed. Microbial fermentation produces large quantities of volatile fatty acids (VFA) that stimulate the development of the absorptive epithelium lining the rumen. Increases in rumen volume and musculature occur in response to feed bulk consumed by calves (Drackley, 2008). Butyrate is a short-chain VFA produced by the anaerobic microbial fermentation of carbohydrates. It is considered the most important VFA for gastrointestinal (GI) tract development as it modulates tissue differentiation, function and proliferation, by providing energy to, and mediating the activity of, colonocytes (Leonel and Alvarez-Leite, 2012). Studies examining the effects of EE supplementation in cattle have found increased total VFA production including an increase in butyrate production (Tricarico et al., 2005, Ranilla et al., 2008, Zilio et al., 2019). If an increase in butyrate production is observed when supplementing dairy calves with EE, improvements in GI tract development, immunity and digestive capabilities could be achieved. Optimising the development of the rumen and post-ruminal tract is one of the greatest challenges when rearing calves and is key to ensuring that they can derive sufficient nutrients from forage and grain-based rations, rather than milk, following weaning.

During the pre-ruminant and transitional stages, the benefits of feeding forages to calves are disputed. The population of cellulolytic bacteria in the rumen cannot increase to sufficient numbers for effective hydrolysis of plant cell walls until the reticuloruminal pH rises to 6.0, which does not typically occur until 10 weeks of age (Anderson et al., 1987). Before that point, the digestion of cellulose is severely limited, with forage intake potentially limiting voluntary intake via the accumulation of undigested material within the rumen. However, consuming small amounts of forages has been shown to stimulate the increase in rumen pH needed for effective microbial activity, to decrease the risk of subacute rumen acidosis in both pre- and post-weaned calves (Laarman and Oba,

2011), and to promote rumination and salivation (Pazoki et al., 2017). Although microbial colonisation of the GI tracts of calves commences almost immediately after birth (Stewart et al., 1988), metagenomic analysis has indicated that microbial populations in the rumen do not stabilize at similar relative abundances to those seen in adult ruminants until 6 to 16 weeks of age, although this is affected by factors including weaning age and calf weight. However, populations are significantly more dynamic in the first year of life than the years following (Amin et al., 2021). Thus, supplementing young ruminants with EE in the transition period, and the remainder of their first year of life, may have beneficial outcomes for forage digestion, given that the rumen microbiome would be expected to be less well equipped for alloenzymatic digestion than it is later in life (Guilloteau et al., 2009). Even in mature cattle, inefficient digestion of cell walls is one of the greatest limitations to their nutrition, with plant cell wall digestibility under ideal conditions less than 65 %, leading to high levels of nutrient excretion given that cell walls comprise 40 – 70 % of the DM content of forages (Van Soest, 1994).

5.2.5 Exogenous enzyme production and classification

Exogenous enzymes commonly used in ruminant nutrition can be split into three categories: fibrolytic, amylolytic and proteolytic. Fibrolytic enzymes include cellulases (endoglucanase, exoglucanase, ß-glucosidases) and hemicellulases (endoxylanase, ß-1,4 xylosidase, glucuronidase, ß-mannosidase, galactosidase), which catalyse the hydrolysis of the polysaccharides cellulose and hemicellulose, respectively (Beauchemin et al., 2003). Amylolytic enzymes catalyse the hydrolysis of starch into glucose and include α-, ß-, and gluco-amylases (Tricarico et al., 2008), and proteolytic enzymes (proteases), catalyse the hydrolysis of protein into amino acids (Eun and Beauchemin, 2005). Other EE used in ruminant nutrition include pectinases (esterases, protopectinases, depolymerases), which catalyse the hydrolysis of pectin (Murad and Azzaz, 2011), and phytases that catalyse the hydrolysis of phytate to release phosphorus and other phytate bound molecules such as amino acids and calcium (Humer and Zebeli, 2015). The two leading methods of EE production are solid-

state fermentation (SSF) and submerged fermentation (SmF), both methods of biotransformation where bacteria (typically *Bacillus* spp.) or fungi (typically *Aspergillus* spp., *Saccharomyces* spp., *or Trichoderma* spp.), are cultivated on a substrate to produce specific enzymes or mixed fermentation products (Sujani and Seresinhe, 2015). SSF and SmF can be differentiated by water requirements, with SSF occurring in the absence of free-flowing water and SmF requiring free-flowing water. SSF typically has higher yields than SmF, produces less waste, has lower production costs and has a lower risk of contamination (Jonathan et al., 2021). Advances in bioengineering have enabled improved efficiency and decreased cost of enzyme production. Genome editing has allowed for the enzyme producing capacity of microbes, such as *Aspergillus niger* and *Trichoderma reesei* to be enhanced. In *A. niger*. the insertion of the glucose oxidase gene at *glaA* and α-amylase loci, using CRISPR-HDR, has quadrupled enzymatic production (Dong et al., 2019), and *T. reesei* strain AR-766 has been genetically modified to produce large quantities of phytase (EFSA, 2025). Notably, genome editing can produce strains with deleterious characteristics, such as mycotoxin production, which are harmful to both humans and livestock, highlighting the need for robust screening before novel strains are used in food processing or for enzymatic production (Dong et al., 2019, Li et al., 2020). To obtain enzymes from SSF or SmF, filtration, solvent extraction and purification steps are required, with the enzyme profile of the fermentation products determined by the microbe strain, cultivation method and substrate composition (Jonathan et al., 2021). Alternatively, the entire fermentation media including substrate, fermentation products and microbes can be harvested and used as feed additives. However, obtaining a homogenous mixture of these components can be challenging, and when recommended inclusion rates of these types of products are low, varying responses from treatments could be caused by a lack of homogeneity, with the actual concentration of enzymes highly varied throughout the product (Krishna, 2005). This is particularly challenging in SSF, where the lack of water makes agitating the fermentation bed difficult, resulting in a heterogenous physical and chemical environment in the fermentation chamber (Krishna, 2005). The activity of enzymatic products is usually quantified by measuring the amount of product produced by the

biochemical reaction that the enzyme catalyses, enabling enzyme activity to be expressed as product produced per unit time, although manufacturers of mixed fermentation products often do not declare enzyme activities (Meale et al., 2014, Parmar et al., 2019). However, the highly controlled lab conditions under which enzyme activities are measured are not representative of the conditions of the rumen (Meale et al., 2014). A fibrolytic enzyme that has cellulase activity of 1500 μ mol/ml/min at 20 °C and pH 7 may have a completely different activity in a rumen at 38 °C and pH 6. To overcome this, testing of enzymatic products using the *in vitro* gas production technique (Menke et al., 1979) can be employed, where the effect of enzyme treatments on digestive efficiency through gas production and fibre degradation is measured under conditions similar to those found in the rumen (Meale et al., 2014).

5.2.6 Justification

Research exploring methods to improve dairy calf performance is not novel. There is an abundance of literature examining biological additives including probiotics, prebiotics and secondary plant metabolites, for the propagation of rumen development and digestive tract health in calves in their pre-ruminant and transitional stages (Diao et al., 2019). The effects of these have sometimes been beneficial, but they are inconsistent (Diao et al., 2019, Reddy et al., 2020). It is possible that pre- and probiotic additives have similar effects as EE, since microbial propagation increases the endogenous enzyme production in the rumen (Uyeno et al., 2015). However, if conditions such as pH and rumen outflow are not favourable to microbes in probiotics, their capacity to produce desirable enzymes would be limited or non-existent (Frizzo et al., 2011). EE supplementation as a feeding strategy for calves, either as a standalone treatment or in combination with other additives, may increase the hydrolytic capacity of the rumen, whilst bypassing the need for favourable conditions for microbial colonisation required for probiotics to be efficacious; potentially enhancing the health, welfare, and performance of calves. Thus, examination of existing research measuring the effects of EE supplementation in calves will

identify any benefits that have already been realised and highlight future research that could be undertaken to further knowledge on the subject.

5.3 Materials and methods

This Systematic review has been conducted following the standards set out in the PRISMA 2020 statement (Page et al., 2021). Prior to database searches, the eligibility criteria listed in Table 5-1 were selected for the inclusion and exclusion of literature in this review. The information sources, search terms, and additional filters used to identify literature are listed in Table 5-2. Initial literature searches were conducted by the primary reviewer, and all articles were imported into EndNote (EndNote X9, 2018). Using the 'Find Duplicates' function, duplicate articles were identified and removed from the reference catalogue. The remaining articles were imported into a Microsoft Excel workbook. The primary reviewer screened all papers according to the selection criteria and 20 % of articles were selected using a random number generator and imported into a blank Excel workbook for title screening by the secondary reviewer. The reviewers agreed on 100 % of the papers to be included, indicating a robust set of selection criteria and a low risk of bias from the primary reviewer. Whole text screening of all papers selected from title screening was performed by the primary reviewer, with 20 % of those randomly selected and imported into a blank workbook for screening by the secondary reviewer. There was 100 % agreement between both reviewers on papers to be included in the review, allowing the primary reviewer to proceed with critical analysis.

Table 5-1. Selection criteria for the inclusion of literature in a Systematic review of exogenous enzyme use in dairy calves.

Criteria	Include	Exclude
Species	Cattle, *Bos taurus* and *Bos indicus*	Any other species
Breed	Dairy and dual-purpose breeds	Beef breeds
Animal age	Animals under 12 months old for the duration of the experimental period	Animals over 12 months old
Treatment	Exogenous enzymes as a standalone treatment or in combination with another treatment	No exogenous enzyme treatment
Experimental parameters	Research that has an animal performance component	Research that uses only blood, tissue, or organ assays
In vitro digestibility research	Trials where the inoculum used is derived from dairy calves under 12 months old	Trials where the inoculum is derived from cattle over 12 months old
Language	Articles that are available in English	Articles that are not available English
Article type and access	All relevant original research articles, short communications, and reviews	Closed access articles where a response from the corresponding author is not received within one month of email contact

Table 5-2. Information sources and search terms used to identify literature for a Systematic review of exogenous enzyme use in dairy calves.

Information source	Search terms	Additional filters	Last date searched
PubMed https://pubmed.ncbi.nlm.nih.gov	(calf OR calves OR preruminant) AND (exogenous enzyme)	Text availability: "Abstract" + "Full text" Species: "Other Animals" Article language: "English"	01.08.2023
Web of Science https://login.webofknowledge.com	Exogenous AND enzyme AND (preruminant OR calf OR calves)	N/A	01.08.2023
ScienceDirect http://www.sciencedirect.com/	(calf OR calves OR preruminant) AND (exogenous enzyme)	N/A	01.08.2023
Google Scholar http://www.scholar.google.com/	(calf OR calves OR preruminant) AND exogenous enzyme	Top 100 hits included	01.08.2023

5.4 Results

The number of research articles qualifying for each of the identification, screening, eligibility and inclusion stages of this review are shown in Fig. 5-1. Of the 17 studies that met the eligibility criteria, 15 found a significant effect of EE as an individual treatment, or in combination with another treatment. These effects are summarised in Fig. 5-2. The treatments used in studies were: four doses of a fungal derived amyloglucosidase (Diazyme) (Morrill et al., 1970); two mixed enzyme products containing 1437 exo-cellulase, 788 endo-cellulase, and 7476 xylanase activities (μmol/ml/min) and 1446 exo-cellulase, 1350 endo-cellulase, and 5091 xylanase activities (μmol/ml/min) (Ghorbani et al., 2007); two doses of *Bacillus lentus* fermentation product containing ß-mannanase (Nabte-Solis, 2008); two doses of phytase (Buendía et al., 2014); two doses of EE + DFM containing 52000 μmol/g amylase, 28000 μmol/g protease, 14000 μmol/g cellulase, 2000 μmol/g lipase, 1000 μmol/g pectinase, *Saccharomyces cerevisiae* and *Lactobacillus spp.* (Kocyigit et al., 2015, Kocyigit et al., 2016); two doses of xylanase (Hernandez et al., 2017a); two doses of xylanase + *S. cerevisiae* (Hernandez et al., 2017b); xylanase at 1.2 UL/ml and xylanase, *Aspergillus spp,* and *Escherichia spp.* (Kapadiya et al., 2019A, Kapadiya et al., 2019B); EFE containing 160 μmol/g cellulase and 4000 μmol/g xylanase and EFE and branched chain VFA containing isobutyrate, isovalerate and 2-methylbutyrate (Liu et al., 2020); two doses of EE + DFM containing protease, cellulase, amylase, lipase, pectinase, *Lactobacillus spp., Bacillus spp., Enterococcus faecium* and *Aspergillus oryzae* (İlhan and Yanar, 2021); an additive containing proteases, lipases, sodium butyrate, organic acids, sorbic acids and silicon dioxide (Szacawa et al., 2021); EFE containing 6×10^6 μmol/kg cellulase and 1×10^7 μmol/kg xylanase, and EFE + DFM containing *S. cerevisiae, Lactobacillus spp., Bifidobacterium bifidium, Pediococcus acidilactici, Bacillus subtilis* and *Enterococcus faecium* (Khademi et al., 2022); two doses of a feed additive (RumEest) containing cellulase, xylanase, ß- glucanase and *S. cerevisiae* (Harani et al., 2022); two EFE containing 8000 IU/kg cellulase and 16000 IU/kg xylanase, and 12000 IU/kg cellulase and 24000 IU/kg xylanase (Anil et al., 2022); and

products containing 0.055 mg/min/mg xylanase, 0.88 mg/min/mg cellulase and *Lactobacillus fermentum*, and xylanase, cellulase, *L. ferementum* and *Pediococcus acidilactici* (Singh et al., 2022). Table 5-3 presents all variables measured in experiments, experimental treatments and animals, and individual effects realised for each study.

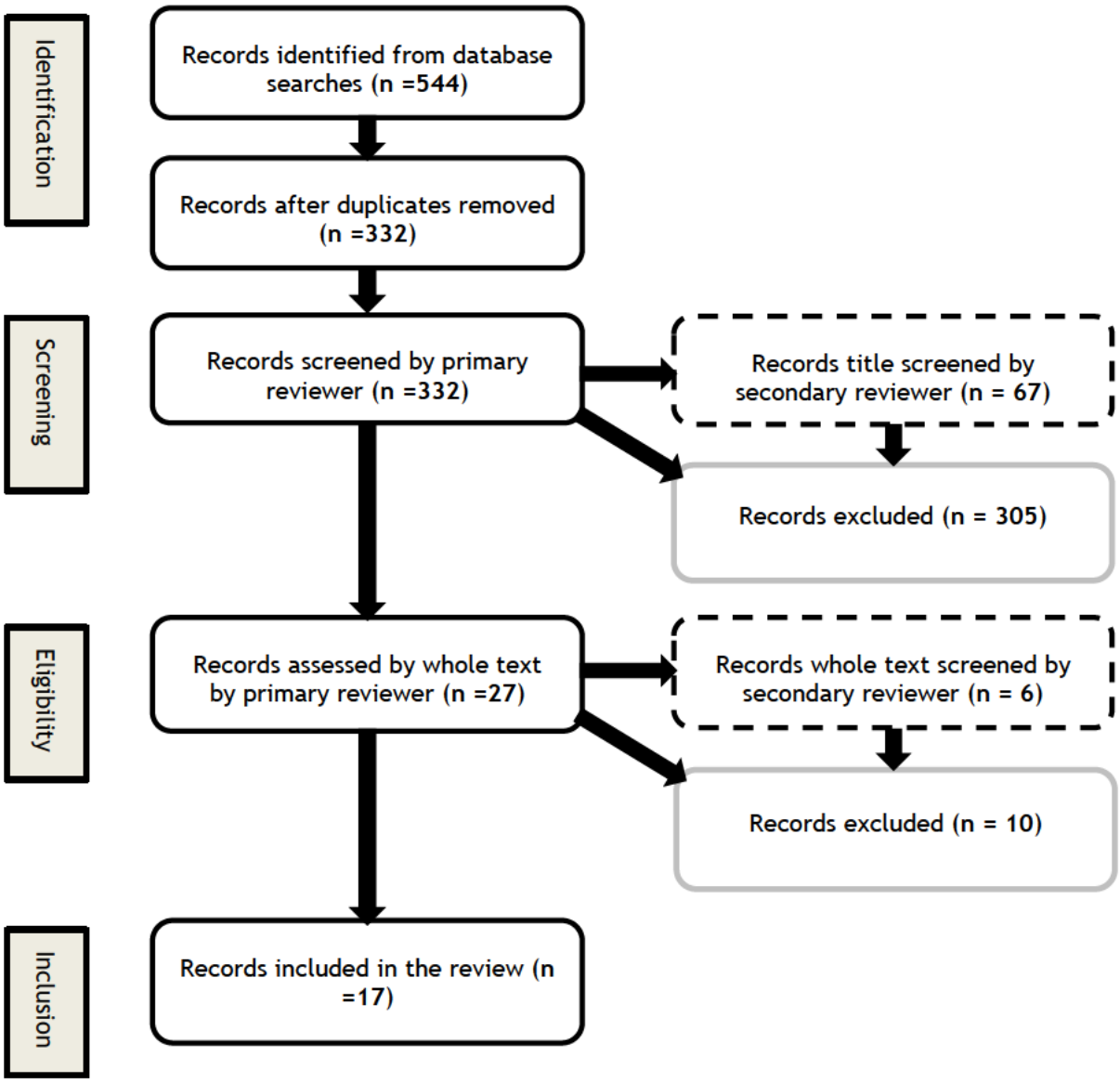


Figure 5-1. PRISMA diagram displaying the number of articles assessed and included in Systematic review of exogenous enzyme use in dairy calves.

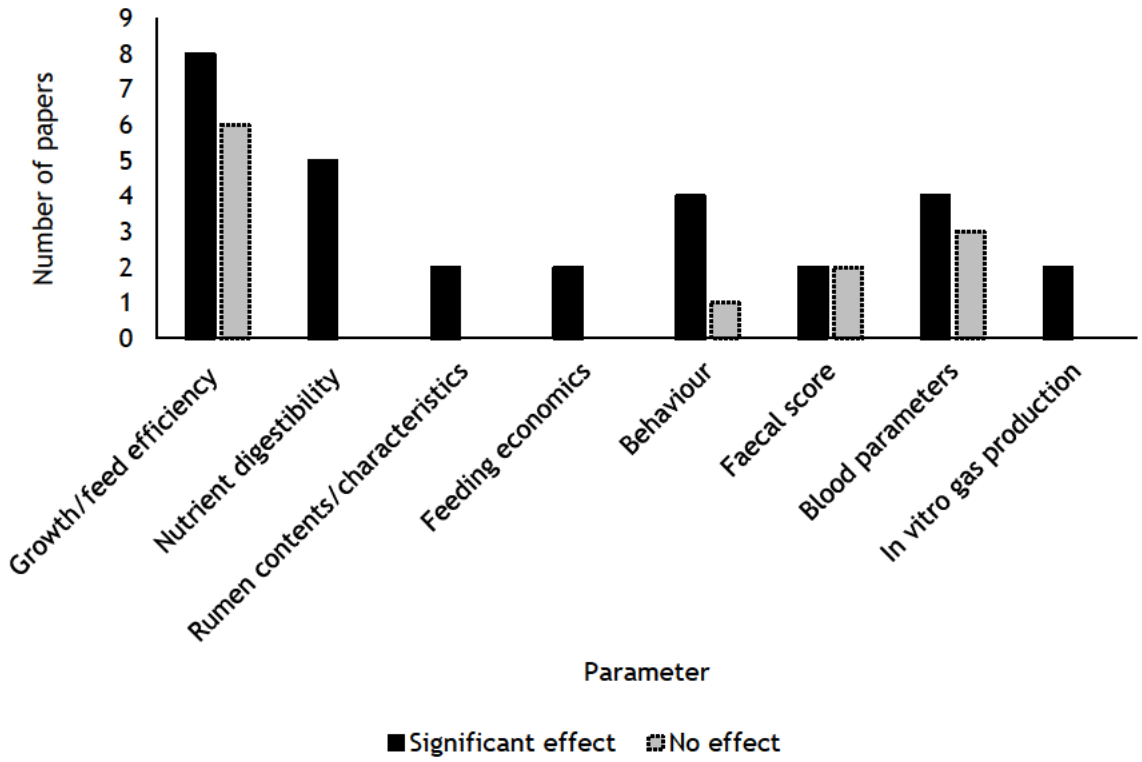


Figure 5-2. Variables measured, and significant effects realised in studies examining the effects of exogenous enzymes in dairy calves. Papers were identified by Systematic review following the PRISMA (2020) framework.

Table 5-3. Summary of papers included in a Systematic review of exogenous enzyme use in dairy calves.

Title	Breed	Age + trial length	Treatment	Animals per treatment	Variable measured	Significant effects	Reference, journal, publisher country
Feeding young calves processed starch combined with an amylolytic enzyme	dairy calves – breed not specified	birth - 6 weeks	amylase + starch in milk replacer (two experiments)	blood trial: 3 growth trial Ctrl: 17 Enzyme: 18	blood parameters ADG	↑ blood glucose no negative effect on ADG from replacing glucose with starch + amylase in milk	Morrill *et al.*, 1970 Journal of Dairy Science USA
Effects of applying exogenous, non-starch polysaccharidases to pre weaning starter concentrate	Holstein	birth - 84 days	EFE (2 products)	6	ADG, BW, feed intake, digestibility	↑ NDF digestibility	Ghorbani *et al.*, 2007 International Journal of Dairy Science

on performance of Holstein calves							Pakistan
Effect of ß-mannanase enzyme addition to soy-containing milk replacers on growth and health of neonatal dairy calves	Holstein	birth - 63 days	ß-mannanase (2 doses) + soy protein in milk replacer	14	ADG, feed intake, FE, body measurements, faecal score, respiratory score, blood, economics of feeding	↑ PUN ↓ rearing cost No negative effect on calf growth or health from the inclusion of soy protein + ß-mannanase in milk	Nabte-Solis, 2008 Cornell University eCommons USA
Effect of an exogenous phytase on growth performance of	Holstein	Undeclared (145 kg weight suggests within criteria) 60-day trial	phytase (2 doses)	8	ADG, feed intake, FE, phosphorus balance, blood	↑ ADG ↓ phosphorus excretion	Buendia *et al.*, 2014 Animal Nutrition and

growing Holstein calves							Feed Technology India
Effect of doses of direct fed microbials plus exogenous fibrolytic enzyme supplementation on growth, feed efficiency ratio and fecal consistency index of Brown Swiss and Holstein Friesian Calves	Brown Swiss and Holstein Friesian	3 days - 6 months	EFE + DFM (2 doses)	Ctrl: 11 10g/d: 7 20g/d: 8	ADG, feed intake, FE, BW, body measurements, faecal score	↓ faecal score	Kocyigit *et al.*, 2015 Indian Journal of Animal Research India
The effect of direct fed microbials plus	Brown Swiss x	3 days - 3 months	EFE + DFM	Ctrl: 11 10g/d: 9	ADG, feed intake, FE, BW, body	↑ ADG ↑ FE	Kocyigit *et al.*, 2016

exogenous feed enzymes on the growth, feed efficiency ratio and some behavioural traits of Brown Swiss x Eastern Anatolian Red F1 calves	Anatolian Red				measurements, behaviour	↑ BW ↑ front shank circumference	Pakistan Journal of Zoology Pakistan
The effect of garlic oil, xylanase enzyme, and yeast on biomethane and carbon dioxide production from 60-d old Holstein dairy calves fed a high concentrate diet	Holstein	60 days	XYL	6 unadapted inoculum donors for *in vitro* GP	GP, GP kinetics, CO_2, CH_4, DMD, pH	↓ CO_2	Hernandez *et al.*, 2017A Journal of Cleaner Production The Netherlands

Effectiveness of xylanase and *S. cerevisiae* as feed additives on gas emissions from agricultural calf farms	Holstein	birth – 60 days	XYL XYL + *S. cerevisiae*	10 10 (*in vitro* study – animal donors for microbial inoculum)	GP, GP kinetics, CO_2, CH_4, DMD, pH	XYL: ↑ rate of GP ↓ CH_4 XYL + *S. cerevisiae:* ↑ CO_2 ↓ total GP ↓ CH_4	Hernandez *et al.*, 2017B Journal of Cleaner Production The Netherlands
Feed intake and feed conversion efficiency of crossbred calves as an effect of fibrolytic microbes and enzymes	Friesian x Kankrej	167 -307 days	EFE EFE+ DFM	4 4	ADG, feed intake, FE	No effect	Kapadiya *et al.*, 2019a Journal of Pharmacognosy and Phytochemistry India

Effect of fibrolytic microbes and enzymes on biochemical blood parameters in crossbred calves	Friesian x Kankrej	167 - 307 days	EFE EFE + DFM	4 4	blood	EFE: ↑ plasma phosphorus ↓ GPT EFE + DFM: ↑ GPT	Kapadiya *et al.*, 2019b Indian Journal of Veterinary Science and Biotechnology India
Effects of branched-chain volatile fatty acids and fibrolytic enzyme on rumen development in pre- and post-weaned Holstein dairy calves	Holstein	15 - 90 days	EFE EFE + BCVFA	10 10	ADG, feed intake, FE, rumen contents and characteristics, blood	EFE and EFE + BCVFA: ↑ ADG ↑ FE ↑ total VFA, acetate, propionate, butyrate ↑ blood GH, IGF-1	Liu *et al.*, 2020 Animal Biotechnology UK

						↑ mucosal GHR, IGF-1R, HMGCS1 ↑ anterior blind sac papillae length and width	
Effect of supplementation of exogenous fibrolytic enzymes on intake, nutrient utilization pattern and economics of feeding in weaned crossbred calves	Jersey	234 - 310 days	EFE (2 doses)	5	ADG, feed intake, FE, digestibility, economics of feeding	↑ DMI/kg BW ↑ OMI/kg BW ↑ CPI/kg BW ↑ DM, OM, CHO, NDF, ADF, hemicellulose, cellulose digestibilities ↓ rearing costs	Anil *et al.*, 2021 Biological forum India

Effect of different levels of direct fed microbials plus exogenous fibrolytic enzyme additives on the growth traits of dairy calves.	Brown Swiss	birth - 6 months	EFE + DFM (2 doses)	6	BW, FE, body cleanliness, faecal score, bedding cleanliness, behaviour	↑ recumbency ↓ fecal score	Ilhan and Yanar, 2021 Journal of Agricultural Sciences Indonesia
A pilot study on the effect of a novel feed additive containing exogenous enzymes, acidifiers, sodium butyrate and silicon dioxide nanoparticles on selected immune indices and body	Holstein Friesian	4 - 8 weeks – 11 - 15 weeks	protease + lipase + butyrate + sorbic acids + silicon	3	ADG, blood	No effect	Szacawa *et al.*, 2021 Journal of Veterinary Research Poland

weight gain of calves							
Supplementation of probiotic and fibrolytic enzymes on growth performance and nutrient utilization in crossbred calves	Jersey x Sahiwal	5/6 - 8/9 months	EFE + DFM (2 doses)	8	ADG, BW, FE, digestibility, behaviour	↑ ADG ↑ BW ↑ rumination time ↑ OM, CP, CF, NDF, hemicellulose digestibilities	Harani *et al.*, 2022 Journal of Animal Research India
Use of exogenous fibrolytic enzymes and probiotic in finely ground starters to improve calf performance	Holstein	birth - 84 days	EFE EFE + DFM	12 12	ADG, feed intake, FE, body measurements, digestibility, rumen VFA, faecal score, behaviour	EFE: ↑ recumbency ↑ NDF digestibility ↓ rumen acetate	Khademi *et al.*, 2022 Scientific Reports UK

						↓ acetate: propionate ↓ heart girth ↓ body barrel (rib region circumference) ↓ NNOB EFE + DFM: ↑ NDF digestibility ↑ rumen propionate ↓ acetate: propionate	
Growth performance, nitrogen balance and blood biochemical parameters on feeding TMR diet	Karan x Friesian	8 - 11 months	EFE + LAB inoculants (2 strains)	6	silage pH, digestibility, feed intake, nitrogen balance, growth, blood	↑ NDF, ADF digestibilities ↓ Silage pH	Singh *et al.*, 2022 Indian Journal of Animal Research

containing sugarcane tops silage supplemented with lactic acid bacteria inoculants and exogenous fibrolytic enzymes in crossbred calves	India

Abbreviations: ADG: average daily gain; EFE: exogenous fibrolytic enzymes; DFM: direct-fed microbials; FE: feed efficiency; BW: bodyweight; PUN: plasma urea nitrogen; XYL: xylanase; GP: gas production; DMD: dry matter disappearance; GPT: glutamic pyruvic transaminase; BCVFA: branched chain volatile fatty acids; VFA: volatile fatty acids; GH: growth hormone; IGF-1: insulin-like growth factor-1; GHR: growth hormone receptor; IGF-1R: insulin-like growth factor-1 receptor; HMGCS1: 3-Hydroxy-3-Methylglutaryl-CoA Synthase 1; NNOB: non-nutritive oral behaviour; DM: dry matter; OM: organic matter; CHO: carbohydrates; NDF: neutral detergent fibre; ADF: acid detergent fibre; CP: crude protein; CF: crude fibre; DMI: dry matter intake; OMI: organic matter intake; CPI: crude protein intake

5.5 Discussion

5.5.1 *In vitro* studies

This review identified two papers that explored the effects of EE in dairy calves *in vitro*, both measuring environmental and digestibility outcomes. Hernandez et al. (2017a) measured the effect of two doses of xylanase (3 and 6 µL/g DM) on the digestibility, gas production (GP), and GP kinetics of calf starter concentrates incubated with rumen inoculum obtained from 60-day old Holstein calves, using the Theodorou et al. (1994) pressure transducer technique. Neither dose significantly affected the total GP from incubation, nor the GP kinetics. Xylanase treatments of 3 and 6 µL/g DM reduced methane production by 29.4 and 33.9 ml/g DM degraded, respectively, compared with controls over a 72-h incubation, but the difference was non-significant. The higher dose of xylanase significantly decreased CO_2 production by 3.77 ml/g DM degraded compared with controls. When performing *in vitro* GP experiments, it is standard practice to adapt the donor animals to the treatments being trialled for at least 14 days prior to inoculum being collected (Yáñez-Ruiz et al., 2016). The calves used as inoculum donors for the trial of Hernandez et al. (2017a) were not adapted to the treatments, potentially limiting the reliability of the results. In the second *in vitro* study included in this review, the same group, Hernandez et al. (2017b), trialled the same doses of xylanase as well as two doses of xylanase in combination with two doses of *S. cerevisiae* (3 µL/g XYL + 2mg/g *S. cerevisiae* and 6 µL/g XYL + 4mg/g *S. cerevisiae*) using the Theodorou et al. (1994) GP technique. In this study, 10 Holstein calves were adapted to each treatment from birth for 60 days prior to inoculum collection, and a significant reduction in methane production was found for all doses of xylanase, and the combination of xylanase with *S. cerevisiae* yielded the lowest proportional methane production of 9 % of total gas produced. A significant increase in the rate of GP was realised from xylanase addition, indicating enhanced digestion speed. Xylanase in combination with *S. cerevisiae* significantly decreased total GP without decreasing the DM disappearance of feedstuffs compared to controls, suggesting

a more efficient fermentation pathway. Hernandez et al. (2017b) postulates that the proportional reduction in methane achieved by treatments could be due to the stimulation of reductive acetogens producing acetate from hydrogen and CO_2, limiting the availability of hydrogen for methane production by methanogens. Confirmation of this theory would have been possible if the VFA profile of fermentation fluid post-incubation had been analysed. The results of Hernandez et al. (2017b) suggest that EE might enhance feed digestibility and reduce the environmental impact of dairy calves, although *in vivo* validation would be required to verify this.

The low yield of studies this review has identified that employ *in vitro* methods to investigate EE, highlights the limitations of such techniques in calves. *In vitro* digestibility trials are common and valuable methods to explore the utility of feed additives prior to *in vivo* studies in ruminants. Although lab-based, the techniques require donor animals for microbial inoculum. In mature cattle, these donor animals are typically fistulated, meaning they can be used to collect rumen contents for innumerable *in vitro* studies. However, for *in vitro* studies examining the effects of feed additives in calves, fistulation of donor animals is problematic given the rapidly changing physiology of calves, which leaves euthanasia and stomach tubing as options for obtaining rumen contents from calves, making such studies less accessible both ethically and practically. The study of Hernandez et al. (2017b) had a robust design, adapting 10 calves per treatment as donor animals for *in vitro* trials. However, *in vitro* effects do not always translate into animal studies and Hernandez et al. (2017b) concludes that further *in vivo* trials are required. Considering the number of calves adapted to each treatment in the study, the sample size would have been large enough to obtain meaningful results for *in vivo* parameters during the 60-day period the calves were fed EE, prior to inoculum collection. Unfortunately, research resources are not infinite and there may have been no scope to collect extra data in this study.

5.5.2 Growth and feed intake

5.5.2.1 Exogenous enzymes as single treatments

This review identified 8 studies that measured the effect of EE as a standalone treatment on the growth and feeding performance of dairy calves, with five of these studies using exogenous fibrolytic enzymes (EFE) as a treatment. Supplementing calves with EFE early in life could enhance their physiological capacity for plant polysaccharide digestion and be beneficial for growth and performance in both pre- and post-weaning stages. However, Ghorbani et al. (2007) trialled two EFE products neither of which had an effect on calf weight gain, feed intake, or feed efficiency. EFE1 was trialled at an inclusion rate of 0.6 ml/kg DM starter and contained exo-cellulase, endo-cellulase, and xylanase activities of 1437, 788, and 7476 μ mol/ml/min respectively; EFE2 trialled at an inclusion rate of 1.9 ml/kg DM starter and contained exo-cellulase, endo-cellulase, and xylanase activities of 1446, 1350, and 5091 μ mol/ml/min respectively. Kapadiya et al. (2019A) measured the effect of feeding calves xylanase with enzyme activity of 1.2 U/ml at an inclusion of 50ml/kg feed and Khademi et al. (2022) measured the effect of feeding an EFE with cellulase and xylanase activities of 6×10^6 U/kg and 1×10^7 U/kg respectively at an inclusion of 2 g/d. Neither study realised a significant effect on calf weight gain, feed intake, or feed efficiency from treatments and calves treated with EFE in the study of Khademi et al. (2022) had significantly decreased heart girth and body barrel measurements.

In the trial of Ghorbani et al. (2007), where the effects of two EFE products were measured using 6 Holstein calves per treatment, from birth for an experimental period of 84 days; enzyme treatments containing cellulase and xylanase at doses of 0.6 and 1.9 ml/kg DM feed were administered in starter concentrates from birth, and both EFE treatments ceased at day 56. Given that neonatal calves will consume little to no concentrate in the first 2 weeks of life, a more reliable method to ensure each calf consumes its treatment would be to administer the EE with milk, at least until the calf is eating consistent amounts

of starter. Ghorbani et al. (2007) does not justify cessation of enzyme treatments at weaning, since calves rely solely on the effective digestion of solid feeds when milk is withdrawn, more pronounced effects of enzymes could be observed in the post-weaning period. The authors suggest the lack in efficacy of the treatments could be due to the enzyme actions being solely against cell wall components. Indeed, cellulases and xylanases predominantly hydrolyse cellulose and hemicellulose, however, calves in this study were not offered forage throughout and the starter concentrate fed was barley and soy based, meaning there was limited dietary fibre for EFE to enhance the digestion of.

Khademi et al. (2022) also administered EFE to neonatal Holstein calves in starter feed for a period of 84 days. Wheat straw was soaked in water containing EFE prior to being included in the starter ration. This is a novel method of enzyme application, whilst moisture is required for enzyme activation, soaking a substrate may limit enzyme attachment, as well as cause some of the sparse soluble nutrients present in the straw to be lost, potentially decreasing its nutritive value. The study found no effect of EFE treatment on average daily gain (ADG) or feed intake but observed a significant decrease in heart girth and body barrel of calves fed EFE treated straw. Khademi et al. (2022) suggests that increased retention time of EFE treated straw may have limited the absorption of minerals required for skeletal growth, causing a decrease in body variables. However, the experimental procedure states that all calves were fed a basal starter containing 7 % chopped wheat straw; it would be expected that calves fed straw that had been treated with EFE would have higher rumen outflow rate from increased neutral detergent fibre (NDF) digestibility. Calves can sort their feeds, so it is possible that soaking wheat straw with EFE increased its palatability leading to increased intake, although composition of daily orts would be required to investigate this. Regardless, feeding young calves structural carbohydrates can aid in rumen development and prevent acidosis from excess non-structural carbohydrate intake. The fibrolytic microbial community in the rumen develops later than the sugar and starch digesting microbes, limiting the ability of calves to clear forages from the rumen (Drackley, 2008). Thus, feeding EFE in a diet containing straw should increase outflow rather than retention

time, providing that the treatment and control calves had the same intake of straw, hence the finding of decreased body barrel could be linked to lower rumen fill.

Kapadiya et al. (2019A) measured the effect of pre-treating Napier grass with xylanase before feeding it to 5-month-old Friesian x Kankrej calves and a non-significant increase was observed for feed efficiency. Notably, Kapadiya et al. (2019A) only used four calves per treatment. A power analysis by Khademi et al. (2022) estimates that a sample size of 12 calves per treatment, $\alpha = 0.05$ and power = 0.80, is required to identify changes in primary response variables including growth and feed intake in calf trials. Thus, the number of calves used by Kapadiya et al. (2019A) would have limited the likelihood of detecting changes in calf performance, should they have been present.

The studies of Liu et al. (2020) and Anil et al. (2022) both found positive effects of EFE supplementation on the growth performance of dairy calves. Liu et al. (2020) found significant increases in ADG and feed efficiency from supplementing 15-day old Holstein calves with 1.83g/d EFE containing cellulase and xylanase activity of 160 and 4000 U/g respectively, over a 75-day period. Distinct from other studies included in this review, treatments were administered to calves in capsules via oesophageal tube. Although this method of administration would not be tenable in most commercial calf rearing units; for scientific studies it is a more reliable way to ensure each calf receives its designated dose, rather than by applying EE to starters or forage. The post-weaning diet of calves in this study was also made up of 60 % alfalfa hay, meaning the EFE had ample dietary fibre on which to work. The enzyme application method and calf diet in this trial offered optimum conditions for EFE to enhance calf performance and, as such, beneficial outcomes were realised. The study of Anil et al. (2022) measured the effect of EFE on more mature dairy calves than those of Liu et al. (2020). Two preparations of powdered *Aspergillus* derived EFE with xylanase and cellulase activities were trialled; EFE1 had xylanase and cellulase activities of 8000 and 16000 U/kg DM, respectively, and EFE2 had xylanase and cellulase activities of 12000 and 24000 U/kg DM, respectively. The powdered enzymes were applied to a total mixed ration (TMR)

made up of chaffed rice paddy straw, oat fodder and concentrates, before feeding to 7-month-old crossbred Jersey heifers. Both EFE significantly increased the voluntary feed intake and feed efficiency of calves over a 75-day growth trial. The composition of the TMR fed to calves in this study was predominantly by-product feeds; if EFE can be utilised to increase the digestibility of such feeds, the sustainability of calf production can be improved through efficient use of crop residues from other food production systems.

A single study examining the effect of phytase supplementation in calves was found. Unlike EFE, the action of phytase is well defined; hydrolysing phytate, the main storage form of phosphorus in cereal grains. Dietary phosphorus has been linked to calf growth rates, however, rumen microbial enzymes do not enable the complete hydrolysis of phytate bound phosphorus, which can lead to poor phosphorus absorption in the small intestine (SI) (Humer and Zebeli, 2015). Phytase is regularly used in the diets of monogastrics to enhance animal performance, gastrointestinal health, and nutrient availability (Holloway et al., 2019). Increased nutrient availability from phytase supplementation is not limited to phosphorus, with increased bioavailability of other nutrients trapped in phytate-complexes, such as calcium and amino acids, also contributing to improved animal performance (Holloway et al., 2019). Buendía et al. (2014) investigated the effects of applying two doses of phytase, 0.012 and 0.024 g/kg feed, to a sorghum-based ration for Holstein calves. The highest dose of phytase caused a significant increase in ADG of calves, and neither dose affected dry matter intake (DMI) or feed efficiency, whilst both doses decreased faecal excretion of phosphorus. This highlights the influence of effective phytate hydrolysis, and consequent phosphorus utilisation, on calf growth performance, as well as reducing the amount of phosphorus excreted, which is advantageous given phosphorus can have negative environmental consequences such as eutrophication.

Two studies identified by this review explored the effects of EE with the inclusion of additional ingredients in calf milk replacers on calf growth and performance. Morrill et al. (1970) trialled the inclusion of 15 ml Diazyme, a fungal-derived amyloglucosidase, combined with gelatinized cornstarch or

expanded sorghum grain as an alternative energy source to glucose in milk replacer. Nabte-Solis (2008) examined the effect of replacing 50 % of whey protein in calf milk replacer with soy protein and ß-mannanase. Both studies found that the growth performance of calves was not affected by the combination of specific nutrients with EE that are expected to hydrolyse them. Starch is a considerably more economical energy source compared with glucose or lactose, however, cannot be utilised efficiently by pre-ruminant calves due to limited amylase production in the abomasum and SI. Morrill et al. (1970) found that when including an amylolytic enzyme resistant to denaturation by the pH of the abomasum, with cornstarch or sorghum that had been processed to expose inter-micellar regions for enzyme action in milk replacer, comparable calf growth performance to that of calves fed conventional milk replacer was achieved. Soy protein is a considerably more economical protein source than whey protein; however, it contains antinutritional factors, such as ß-galactomannans, an insoluble non-starch polysaccharide that can inhibit the absorption of nutrients in the SI (Castanon et al., 1997). Nabte-Solis (2008) found that replacing half of the whey protein in conventional milk replacer with soy protein and two doses of ß-mannanase, 20000 U/kg and 50000 U/kg, derived from the fermentation products of *Bacillus lentus*, had no negative effect on feed intake, ADG, or skeletal parameters of Holstein calves, compared with calves fed milk replacer containing 100 % whey protein. Calves fed soy protein in milk replacer had significantly increased feed intake in the post-weaning period, however, this was independent of enzyme treatment.

The majority of studies utilising EE as a standalone treatment found beneficial effects for calf growth or feeding performance, this includes the studies of Morrill et al. (1970) and Nabte-Solis (2008) where equal calf performance to the control can be considered a positive outcome. These trials have demonstrated that EE can be used efficaciously in calves, when an enzyme with known actions is selected to target a specific substrate, for a specific purpose.

5.5.2.2 Exogenous enzymes in combination with another treatment

This review identified nine studies that measured the effect of EE in combination with another treatment on calf growth and performance. Of these studies, six examined the effect of feeding calves EE with direct fed microbials (DFM). Although EE treatment has been shown to positively manipulate the rumen microbiome of mature cattle (Meale et al., 2014), the inclusion of DFM might influence the size and direction of the effects, which could be advantageous in calves, given the impact of rumen development and overall gut health on calf performance. However, four of the six studies measuring the effect of EE + DFM did not find any significant effect of treatment on calf growth or feed intake.

İlhan and Yanar (2021) measured the effect of feeding Brown Swiss calves two doses: 10 and 20 g/d, of a powdered product containing enzymes and DFM. Enzymes included protease, cellulase, amylase, lipase, and pectinase, however, their activities were not declared. DFM contained *Lactobacillus* spp., *Bacillus* spp., *Aspergillus oryzae,* and *Enterococcus faecium*. Treatments were administered from birth until six months of age. Calves treated with both doses of EFE + DFM had numerically higher ADG and feed efficiencies, with calves on the lower dose achieving a feed efficiency 64.2 % higher than control calves. However, this was not statistically significant suggesting that the study was underpowered.

As well as the standalone EFE treatment trialled in the study of Khademi et al. (2022), the effect of EFE + DFM containing *S. cerevisiae, Lactobacillus* spp., *Bifidobacterium bifidium, Pediococcus acidilactici, Bacillus subtilis,* and *Enerococcus faecium*, on calf growth and feed intake was measured. No increases in ADG, feed intake or efficiency were observed. However, calves fed EFE + DFM did not have the significantly decreased heart girth or body barrel that was observed in calves fed only EFE treated straw. Interestingly, Khademi et al. (2022) trialled DFM without EFE as a treatment, which resulted in significant increases in ADG, total BW and feed efficiency. These results suggest that the effects of EFE when combined with DFM, might inhibit beneficial growth

and feeding performance outcomes from DFM treatment. Calves fed EFE and EFE + DFM had numerically lower feed intakes, which could have caused poorer growth performance. The results of Khademi et al. (2022) indicate that feeding dairy calves DFM and straw pre-treated with EFE is not beneficial for calf growth or feed efficiency. Kapadiya et al. (2019A) also trialled the effect of combining an EFE treatment with DFM containing *Aspergillus* spp. and *Escherichia* spp., on the growth and feed efficiency of 5-month-old Friesian x Kankrej calves. Similar to the outcomes of the standalone EFE treatment, EFE + DFM led to numerical improvements in calf ADG and feed efficiency. Had the trial used more than four calves per treatment, greater evidence for the efficacy of treatments would be available.

Kocyigit et al. (2015) trialled the effects of two doses of EE + DFM on the growth and feed efficiency of Brown Swiss and Holstein Friesian calves, from birth until six months of age. The EE used in this trial is referred to as fibrolytic, however, its activity was predominantly amylolytic and proteolytic: 52,000 U/g amylase; 28,000 U/g protease; 14,000 U/g cellulase; 2000 U/g lipase; 1000 U/g pectinase; the DFM contained *S. cerevisiae* and *Lactobacillus* spp. Calves were split unevenly between control (n = 11), 10 g/d (n = 7), and 20 g/d (n = 8). This uneven allocation to treatment is not clarified; calf mortality is a plausible cause. The study used 16 Brown Swiss and 10 Holstein Friesian calves, and the author states that calves were randomly allocated to treatment, however, the breed of calves in each treatment is not declared. Unsurprisingly, significant differences were found between body weights and measurements of Holstein Friesian and Brown Swiss calves. However, no significant differences for ADG, body measurements, or feed efficiency were observed from treatments. Calves fed 10 g of EE + DFM had numerically higher weight gains and body measurements than control calves throughout the trial. However, without knowing the breeds of calves in each treatment it is impossible to know whether these increases were due to breed or treatment. Although obtaining a sufficient number of calves close enough in age may have been the cause of using two breeds in this study, breed differences between Holstein Friesian and Brown Swiss calves make ascertaining any conclusions about the efficacy of EE + DFM

difficult. However, in a further study, Kocyigit et al. (2016) goes on to trial the 10 g/d EE + DFM on a uniform sample of calves – Brown Swiss x Eastern Anatolian Red, from birth until three months of age. In this study calves fed EE + DFM had significantly greater weights at weaning and three months old, significantly improved feed efficiency between birth and weaning, and a significant increase in front shank circumference compared with control calves. Given the numerical increases in weight gain and body measurements observed by Kocyigit et al. (2015), it is likely the EE + DFM treatment was effective, but breed differences confounded the result. When utilising calves of the same lineage, Kocyigit et al. (2016) observed significant improvements in the growth and feed efficiency of calves fed 10 g/d EE + DFM. This highlights the importance of minimizing variations, such as breed in calf trials, whereby confounding factors can mask the efficacy of experimental treatments.

Harani et al. (2022) trialled the effects of two doses of RumEest-ESF (Neospark drugs and chemicals ltd), a product containing active *S. cerevisiae* at 5 x 10^9 CFU/g and cellulase, xylanase, and ß- glucanase, on the growth and feed efficiency of five-month-old Jersey x Sahiwal calves. Eight calves per treatment were fed 0, 10, or 15 g/d RumEest-ESF, applied to concentrate feed an hour before feeding, whilst calves had *ad lib* access to hybrid Napier grass. A positive dose-response from treatment was observed, with calves administered RumEest-ESF having significantly increased ADG and total weight gain. There was a non-significant improvement in feed efficiency with increasing dose, suggesting that the combination of yeast and EFE may improve the digestibility of feedstuffs, possibly through the propagation of the rumen microbiome and release of cell bound sugars and proteins, leading to the significant improvements observed for calf growth.

This review identified a single study that used EFE as a component of a silage additive. Singh et al. (2022) ensiled sugar cane tops with EFE containing xylanase and cellulase activities of 0.055 and 0.88 mg/min/mg respectively, and two types of lactic acid bacteria (LAB) inoculum from *Lactobacillus fermentum* and *Pediococcus acidilactici*, for 30 days prior to feeding to 8-month-old Karan x Friesian calves. The use of EE as silage additives for forages fed to mature cattle

has been reviewed by Taye and Etefa (2020), with positive outcomes for growth and milk production in cattle observed from pre-treating silage with EE. Notably, using EE as a silage additive allows for a considerably longer timeframe for enzyme action, compared with direct feeding of EE, allowing for improved hydrolysis of plant polysaccharides and potential synergism between EE and LAB in the anaerobic fermentation phase of ensiling. Thus, positive performance outcomes from enhanced silage quality could be expected. However, in the study of Singh et al. (2022), although EFE + LAB treatments made better silage with a significantly lower pH and higher NDF and acid detergent fibre (ADF) digestibilities, this did not affect the growth or feed intake of calves, compared with control calves fed a silage treated with urea, molasses and NaCl. Unfortunately, EFE and LAB were not trialled as individual treatments in this study, so it is impossible to discern if the beneficial outcomes for silage quality were from the EFE or LAB or synergy between them.

Liu et al. (2020) measured the effect of 1.83 g/d EFE, containing 160 U/g cellulase and 4000 U/g xylanase, in combination with branched-chain volatile fatty acids (BCVFA) on calf growth and feed efficiency. The BCVFA contained 2-methylbutyrate, isobutyrate, and isovalerate. The production of VFA from feed degradation by microbes stimulates rumen development in calves, with butyrate playing a key role in rumen epithelial cell proliferation, pH homeostasis, and ketogenesis (Niwińska et al., 2017). Thus, supplementing calves with EFE to stimulate microbial propagation and feed degradation, and BCVFA to enhance rumen development, could have synergistic effects for calf performance. Liu et al. (2020) observed that supplementing Holstein calves with EFE + BCVFA through the pre- and post-weaning stages significantly improved DMI, ADG, and the feed efficiency of calves. With the results for DMI and ADG numerically higher, and feed conversion ratio numerically lower, than results from calves administered BCVFA or EFE as a standalone treatment. A statistical interaction was not observed between BCVFA and EFE, suggesting an additive response. Liu et al. (2020) demonstrated that supplementing calves with EFE alone or in combination with BCVFA can lead to significant improvements in calf performance. This study yielded more beneficial results than most studies that

combined EFE with DFM, suggesting that EFE + BCVFA might be more advantageous for calf performance than EFE + DFM.

Szacawa et al. (2021) measured the effect of a feed additive containing microbial derived proteases and lipases, sodium butyrate, organic and sorbic acids, and silicon dioxide, on the ADG of Holstein Friesian calves, and concluded that the additive might promote digestive tract maturation and health. A large, but non-significant increase in ADG observed from treatment suggests the study was likely under-powered. Six calves were randomly allocated into control and treatment groups and each group was housed in a single pen for seven weeks; the age range of calves at the beginning of the trial was between four and eight weeks old. As calves do not have linear growth curves, having such a large age gap confounds results. Control calves had an ADG of 20 ± 167 g compared with the 231 ± 130 g of calves treated with the feed additive. This result was non-significant, likely caused by the study being under-powered, with growth confounded by age and treatment. The author does not declare individual ages of calves, so it is unclear whether the treatment had an effect or if the calves in that group were just older. A more robust experimental design is required to explore any effect this additive may have on calf performance.

Of the nine studies trialling the effect of EE in combination with another treatment, three found a significant improvement in the growth and or feed efficiency of dairy calves. Notably, most studies observed non-significant improvements in calf performance from treatment, and no reductions in growth or feed efficiency were declared.

5.5.3 Nutrient digestibility

Five of the papers identified by this review measured the effect of EE alone or EE in combination with another treatment, on nutrient digestibility in calves. All papers utilised fibrolytic enzymes and all of them reported significant improvements in the digestibility of NDF. Given that the primary mode of action of EFE is to hydrolyse plant polysaccharides, this indicates that the EFE were performing their intended function, although this did not always translate into improved calf performance. Khademi et al. (2022) realised an increase in NDF

digestibility from feeding calves wheat straw treated with EFE and EFE + DFM, but treatments did not significantly affect the digestibility of ADF, DM, ether extract, or crude protein (CP). Dietary non-fibre carbohydrate levels increased from soaking wheat straw with EFE, suggesting the partial hydrolysis of hemicellulose. The weakening of lignin-cellulose-hemicellulose complexes can facilitate enhanced fibre digestion by rumen microbes, providing greater opportunity for microbial attachment to substrates. However, in the study of Khademi et al. (2022), increased NDF digestibility did not enhance calf performance. Similarly, the pretreatment of sugarcane top silage with EFE and two types of LAB inoculum in the study of Singh et al. (2022) significantly increased the NDF and ADF digestibilities of the silage, whilst having no effect on DM, organic matter (OM), CP or ether extract digestibilities. However, the increase in NDF and ADF digestibility did not significantly affect the feed intake or growth performance of calves. The addition of one of the two types of EFE to neonatal calves starters led to increased NDF digestibility at week four in the trial of Ghorbani et al. (2007). However, this effect was transient and was not observed at week eight. The other EFE trialled, which had higher cellulase and lower xylanase activity, did not affect NDF digestibility for the first eight weeks of the trial, but when treatment was withdrawn a significant decrease in NDF digestibility was observed. Both Ghorbani et al. (2007) and Khademi et al. (2022) used spot faecal sampling for analysis of the digestibility of nutrients. The different digestibilities and passage rates of feeds through the digestive tract makes spot sampling an unreliable method of faecal collection for digestibility analysis, especially given the small amount of faeces that can be obtained from manual stimulation of a neonate rectum. Total faecal collection is the gold standard, however, is not always feasible (Morris et al., 2018).

Anil et al. (2022) found that supplementing weaned calves with two doses of xylanase and cellulase significantly increased the digestibility of OM, DM, ADF, NDF, cellulose, and hemicellulose, but had no effect on ether extract or CP. The addition of EFE to a TMR fed to calves increased the voluntary intake of CP, DM, and OM, leading to an increase in feed efficiency. This highlights the benefits that can be realised from increasing the hydrolytic capacity of the rumen and

stimulating feed intake from the addition of EFE in calf diets. The calves in this trial were weaned, so the utility of enhanced fibre digestion may have been more impactful than in pre-weaned calves on milk and high starch starter feeds. Harani et al. (2022) measured the effect of two doses of EFE in combination with *S. cerevisiae*, on the digestibility of nutrients in weaned calves. Significant increases in the digestibility of OM, CP, NDF, and crude fibre. No effect was observed for the digestibilities of DM, ether extract, ADF, cellulose, or nitrogen free extract. The preferential hydrolysis of hemicellulose by EFE is highlighted by these results, and fibre degradation may have been enhanced by yeast promoting cellulolytic microbial growth in the rumen. The increased digestibilities from treatments led to significantly higher total digestible nitrogen and digestible CP in the diets of calves whilst DMI was unaffected, indicating that calves fed EFE + *S. cerevisiae* received a higher plane of nutrition compared to control calves. Both Anil et al. (2022) and Harani et al. (2022) measured nutrient digestibility using the robust method of total faecal collection over periods of six and seven days, respectively, meaning the results obtained from these trials are more reliable representations of digestibility than studies that use spot sampling of faeces for analysis.

Regardless of application method, dose rate, or combination of EFE with a microbial treatment, all papers identified by this review that measured nutrient digestibilities observed a significant increase in NDF digestibility from EFE treatment. This provides strong evidence that EFE are effective at hydrolysing plant cell walls, however, it is difficult to conclude how useful this is in calf diets, given that increased NDF digestibility often did not significantly improve calf growth or feed efficiency. Studies being under-powered could mean that a small effect on growth and feed efficiency from increased NDF digestibility was statistically undetectable.

5.5.4 Rumen characteristics and contents

A successful transition from pre-ruminant to ruminant can be characterised by the development of the rumen and the microbial community that resides therein, enabling VFA and microbial protein production from plant materials, for

the growth and maintenance of the weaned calf. Assessing calf rumen contents and characteristics is invasive, requiring stomach tubing or euthanasia, which can be problematic ethically and might explain why only two studies identified by this review quantified these parameters. Nonetheless, these measurements can provide explanations for responses to treatments and are especially valuable for evaluating the effects of EFE, given their proposed mechanism of action is on the hydrolytic capacity of the rumen. Khademi et al. (2022) stomach tubed neonatal calves fed EFE and EFE + DFM and found a significant increase in molar acetate and the acetate to propionate ratio from both treatments, EFE + DFM also significantly increased propionate concentration. Increased acetate is in accordance with the improved NDF digestibility observed from treating wheat straw with EFE, as acetate is the main product of fibre digestion. Acetate is positively associated with rumen volume increases, however, excessive amounts can negatively affect rumen papillae growth (Kazana et al., 2022). Liu et al. (2020) found that administering neonatal calves both EFE and EFE + BCVFA significantly increased total rumen VFA concentrations, and the molar concentrations of acetate, propionate, and butyrate. EFE and EFE + BCVFA significantly increased the length and width of papillae in the anterior dorsal blind sac. Notably, BCVFA treatment increased papillae length and width elsewhere in the rumen, as well as total rumen weight, but no interaction between EFE and BCVFA was observed for those parameters. Analysis of functional gene expression revealed significant increases in mRNA expression of 3-hydroxy-3-methylglutaryl-CoA synthase 1, insulin-like growth factor-1 (IGF-1) receptor, and growth hormone (GH) receptor in the rumen mucosa of calves treated with EFE and EFE + BCVFA. These results demonstrate that treatments enhanced rumen function and development and are explanatory factors for the improved growth rates and feed efficiencies of calves treated with EFE and EFE + BCVFA.

5.5.5 Feeding economics

The use of feed additives to enhance cattle health and performance is intrinsically linked to farm economics, with producers unlikely to invest in

additives that do not provide a return. Replacement heifer rearing is expensive in dairy operations, cost analysis by Boulton et al. (2017) demonstrates that the average cost of rearing a heifer from birth to first calving is £1819, with the daily cost of rearing between birth and weaning calculated at £3.14 compared to £1.65 in the post-weaning period. The increase in feed efficiency and performance that has been realised by some studies in this review should lead to improvements in the economics of calf rearing. However, only two studies performed cost analyses. Anil et al. (2022) found the inclusion of two doses of EFE to a post-weaned calf TMR increased the feed intake and efficiency of crossbred Jersey heifers. This led to a decrease in feed cost of 4.73 and 3.56 %, with the lower dose of enzyme causing the greatest decrease in cost, although the authors did not perform statistical analysis on those figures. Considering that plant cell wall digestibility is less than 65 % under ideal conditions (Van Soest, 1994), and developing calf rumens are unlikely to provide ideal conditions for cell wall digestibility, EFE may be used as a method to unlock nutrients in plant polysaccharides, increasing nutrient availability per kg feed, leading to improvements in both performance and the economics of feeding. Nabte-Solis (2008) found that replacing 50 % of whey protein in conventional milk replacer with soy protein and ß-mannanase had no negative effect on calf performance and significantly decreased the cost of calf rearing by 30 %. This demonstrates the potential efficacy of EE to enhance the nutritive value of alternative feedstuffs for calves, and the economic benefit that can achieve. Performing cost analyses develops the utility of research outcomes to producers and helps relate academic findings into industry applications; however, such analyses would require more robust datasets than the majority of the studies included in this review have produced.

5.5.6 Behaviour

Behaviour is a key indicator of calf health and welfare. Four studies measured the effect of EE supplementation on behavioural parameters, three of which found positive effects. İlhan and Yanar (2021) found a significant increase in the percentage of time Brown Swiss calves fed EFE + DFM spent in recumbency. Rest

is an inelastic behavioural requirement for the health and productivity of all cattle, regardless of maturity. Limited resting time and poor growth rates are highly correlated, with healthy calves spending approximately 16 hours per day laying down (Wilson et al., 1999). Thus, the increased recumbency observed from EFE + DFM treatment is positive and could be indicative of calves experiencing positive affective states. If the rumen of calves is not sufficiently developed to derive the nutrient requirements of calves at weaning, they are more likely to experience the negative affective state of hunger and associated behavioural responses, which include decreased resting (Scoley et al., 2019). Although İlhan and Yanar (2021) did not realise significant improvements in calf growth or feed efficiency, EFE + DFM may have enhanced rumen development, allowing for a smoother transition from milk to solid foods, indicated by increased resting time of calves. Khademi et al. (2022) also realised an increase in recumbency of calves fed EFE, and both EFE and EFE + DFM treatments decreased the amount of non-nutritive oral behaviour (NNOB) displayed by calves. Calves are highly motivated to suckle, and unrewarded feeding behaviour, such as sucking pen fixtures or cross suckling other calves can be indicative of negative affective states (Jensen, 2003). Cross suckling can also have damaging consequences for the udder health of other animals, especially if the behaviour persists into adulthood (Jensen, 2003). Measuring the amount of time calves spend performing NNOB is a valuable welfare indicator that can readily be performed on farm and in research. The study of Khademi et al. (2022) was the only trial identified by this review that measured NNOB in calves; their results provide evidence that EFE and EFE + DFM can reduce unrewarded feeding behaviours.

Kocyigit et al. (2016) found that supplementing Brown Swiss x Eastern Anatolian Red calves with EFE + DFM numerically increased the amount of time calves spent in recumbency, although this was non-significant, and no effects were observed for time spent standing, feeding, or drinking water. Harani et al. (2022) found no effect of supplementing EFE + *S. cerevisiae* on the amount of time calves spent recumbent, however, a significant increase in the amount of time calves spent ruminating was observed. In mature cattle, both EFE and yeast

treatments have increased rumination time (Silva et al., 2016, Salvati et al., 2015). Rumination is the most readily available marker of rumen function, with the increase in rumen activity from EFE + *S. cerevisiae* treatment likely correlated with the increases in ADG and BW observed by Harani et al. (2022). Unlike the studies of İlhan and Yanar (2021), Khademi et al. (2022) and Kocyigit et al. (2016), where calves were enlisted into experiments as neonates, the calves used by Harani et al. (2022) were 5 – 6 months old, so it is not possible to draw conclusions as to the effect of EFE + *S. cerevisiae* on rumen development from these results. Notably, all studies used visual observations to collect behavioural data, although considerably cheaper than remote monitoring technology, visual observation protocols are vulnerable to human error and subjectivity, as well as the risk of altered animal behaviour caused by a human presence and extrapolating data collected in discrete timeframes over an entire experimental period. Visually collected behaviour data is valuable, however, greater insights into calf behaviour can be achieved using sensors, though the cost of such technology can be prohibitive.

The majority of studies that measured the effect of EE on calf behaviour discovered beneficial outcomes. Calf welfare is an increasingly important topic across social and economic agendas, with consumer concerns surrounding dairy calf rearing frequently featuring in the media. Although further research is required, the studies identified by this review suggest that EFE supplementation could be an effective strategy to improve calf comfort, especially around weaning.

5.5.7 Calf health status

5.5.7.1 Faecal and respiratory health

Dairy calves are highly susceptible to disease while their immune systems are developing. Diarrhoeal diseases, which can be caused by infectious and non-infectious factors, are the most important diseases of neonatal dairy calves, negatively affecting calf performance and welfare, and being responsible for more than half of neonatal calf mortality (McGuirk, 2008). Four studies

identified by this review measured the effect of EE or EE + DFM on the faecal score of calves, a single study measured the effect of EE on respiratory score. Kocyigit et al. (2015) and İlhan and Yanar (2021) found a significant reduction in the faecal scores of calves fed EFE + DFM, indicating a lower incidence of diarrhoea. Whilst Khademi et al. (2022) did not find a significant effect of EFE or EFE + DFM on calf faecal score. The probiotic effects of DFM might positively influence the colonisation of microbiota in the calf digestive tract, decreasing its susceptibility to pathogens. The use of DFM as prophylactic and therapeutic treatments for calf diarrhoea has been widely investigated, frequently finding positive effects that suggests they may be a suitable alternative for antibiotics in treating diarrhoeal diseases in calves (Davies et al., 2022). However, the utility of EE, which may also promote gut health and the actions of DFM, whilst improving nutrition allowing for the maintenance of a healthy immune system, has not been thoroughly investigated and requires further research. In the study of Nabte-Solis (2008), replacing 50 % of whey protein in milk replacer with soy protein and ß-mannanase did not affect calf faecal or respiratory scores. The anti-nutritional factors present in soy protein can cause nutritional diarrhoea in calves, thus improving their digestion using EE could reduce this risk. Notably, calves fed soy protein without ß-mannanase did not have significantly different faecal or respiratory scores, so it is not possible to conclude that enzyme inclusion had a positive impact on calf health.

5.5.7.2 Blood biochemistry and haematology

Of the seven papers identified by this review that measured blood biochemistry and haematology variables, four found that EE or EE in combination with another treatment, had a significant effect on blood parameters in calves. When determining optimal amylase dose and processing method for starch to be included as an alternative energy source in calf milk replacer, Morrill et al. (1970) found that all doses (6, 12, 18 ml Diazyme) induced significant increases in blood glucose levels for several hours when sorghum was gelatinized. Amylase treatment did not significantly affect blood glucose when unprocessed and steamed sorghum were fed to calves. This preliminary experiment enabled

Morrill et al. (1970) to ascertain that Diazyme was most effective at enhancing the digestibility of gelatinized starch, leading to positive outcomes for calf performance in the subsequent growth trial. Nabte-Solis (2008) found that calves fed ß-mannanase and soy protein in milk replacer had significantly higher plasma urea nitrogen (PUN), indicative of increased protein availability. Soy protein can cause inflammatory responses, however, haptoglobin levels between calves were similar, indicating that treatment did not induce inflammation.

Kapadiya et al. (2019B) measured a significant increase in phosphorus and a significant decrease in glutamic pyruvic transaminase (GPT) in the plasma of Holstein x Kankrej calves fed EFE, and a significant decrease in GPT in calves fed EFE + DFM. However, all blood parameters were within normal ranges, and the author states that this means treatments had no adverse effects on the performance of calves. Notably, treatments had no effect on animal performance, as described by Kapadiya et al. (2019A). Singh et al. (2022) did not realise any significant differences in the blood GPT, PUN, calcium, glucose, creatinine, or glutamic oxaloacetic transaminase in calves fed sugarcane top silage inoculated with LAB + EFE. In research where by-product feeds with low nutritional content are fed, measuring blood metabolites is a valuable method to identify deficiencies. No such deficiencies were observed by Singh et al. (2022). In the study of Buendía et al. (2014), phosphorus retention was increased by feeding Holstein calves phytase. However, this did not affect serum levels of phosphorus; hyperphosphatemia is rare in calves, as phosphorus homeostasis is achieved at greatly varying levels of phosphorus availability (Challa et al., 1989). The feed additive containing protease, lipase, butyrate, acidifiers, and silicon, trialled by Szacawa et al. (2021) did not modulate haematological parameters in calves. No effect on leukocytes, lymphocytes, granulocytes, and monocytes, were observed. It is possible that one or more of the components in the feed additive could influence calf immunity, but the small (n=3) sample size, and 4-week age disparity between calves, was likely prohibitive for measuring the effects of treatment. Calves administered EFE and EFE + BCVFA in the study of Liu et al. (2020) had significantly higher levels of GH and IGF-1 in their blood. This corresponds with the increased gene expression in rumen mucosa and can

be associated with the improvements in ADG and higher concentration of rumen VFA, which can positively affect the amount of GH and IGF-1 produced. The study of Liu et al. (2020) demonstrated positive effects on calf performance with EFE and EFE + BCVA, and performed thorough analysis to determine treatment actions and interactions. Economic limitations do not always allow such extensive investigations. However, comprehensive understanding of the mechanisms of EE at cellular, tissue, organ, organ system, and organism levels, will enhance their utility in calf rearing, as well as allowing for more targeted approaches for the development of novel products.

5.6 Conclusion

This review highlights the broad spectrum of actions that EE supplementation can have in dairy calves. Papers identified by this review realised beneficial outcomes for: calf growth, feed intake, digestibility, behaviour, health, development, and the economics of rearing. However, mirroring the research into EE use in mature cattle, there are inconsistencies in the findings of research that has taken place in calves. Although these differences could be caused by lack of statistical power, heterogenous outcomes from EE treatment will not encourage industry uptake, and further research is required to define outcomes from treatments, and the mechanisms of action that underpin these outcomes. Given the need for enhanced sustainability in the dairy industry, the findings of this review suggest that further research to define the efficacy of enzymatic products in calves is warranted.

Chapter 6. Effects of an *Aspergillus niger* fermentation product and yeast mannan-rich fraction on dairy calf performance and rumen development

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6.1 Abstract

This Research Paper tested the hypothesis that the dietary inclusion of a fermentation product of *Aspergillus niger* (ANP), alone, or in combination with a mannan-rich fraction derived from *Saccharomyces cerevisiae* cell wall (MRF), would enhance dairy calf performance and rumen variables. Thirty Holstein-Friesian bull calves (age 19 ± 5 d) were obtained from a single farm and randomly assigned according to days of age to one of three dietary treatment groups and one of two cull dates, experimental day 27 (immediately prior to weaning) or experimental day 70. Dietary treatment groups were: CTRL, no treatment; ANP, 3 g/d ANP; MRF, 3 g/d ANP and 2 g/d MRF. Calves were housed and fed individually and had *ad libitum* access to starter concentrates, water, and straw. Throughout the trial, ANP calves consumed a greater quantity of solid starter feed than CTRL and MRF calves. Body weight, average daily gain, apparent total tract dry matter digestibility and faecal scores were unaffected by treatment. Calves fed ANP and MRF had improved feed conversion efficiency compared with CTRL in some weeks, but this was not consistent throughout the pre-weaned and weaned periods. Calves fed MRF had a decreased acetate to propionate ratio compared with CTRL pre-weaning, and ANP had a lower acetate to propionate ratio and higher propionate proportion of total VFA compared with CTRL post-weaning. At both culling timepoints, ANP and MRF had thicker strata basale, spinosum and granulosum in their rumen papillae compared with CTRL.

The inclusion of ANP and ANP + MRF could favourably alter rumen fermentation and increase the metabolic and absorptive capacity of papillae in transition stage dairy calves butno consistent effects of treatment on calf growth or feed efficiency were observed in this trial.

Keywords: dairy calf; rumen development; papillae; *Aspergillus niger*; *Saccharomyces cerevisiae*

6.2 Introduction

The health and nutrition of dairy calves is inherently linked with their performance as youngstock, as well as having lasting effects on their productivity once they enter the dairy herd, with the dividends of enhanced calf care often observable in improved lifetime production (Van Amburgh et al., 2014). The inclusion of fungal fermentation products in calf diets is a nutritional strategy that can have beneficial outcomes for health, growth and development (Cangiano et al., 2020, Yerby et al., 2025B). These products can contain a suite of bioactive compounds, such as enzymes, mannans, glucans, organic acids and alkaloids, which can positively manipulate the gut microbiome, enhance immune function, increase the digestibility of feedstuffs and propagate the development of the gastrointestinal (GI) tract (Thomas et al., 2013, Amin and Mao, 2021). Synergen® (Alltech Inc., Kentucky, US) is a commercially available product of the solid-state fermentation of *Aspergillus niger* (ANP), containing residual enzyme activities. Supplementing mature cattle with *Aspergillus* fermentation products has demonstrated beneficial effects on digestibility and production responses, with effects widely attributed to the products acting as prebiotics and digestibility enhancers (Tricarico et al., 2005, Sosa et al., 2022). Feeding *Aspergillus* fermentation products to calves has led to younger weaning ages, increased volatile fatty acid (VFA) production (Beharka et al., 1991), increased feed intake and improved feed conversion efficiency (FCE) (Anil et al., 2022). Although some studies have found limited or no effects of *Aspergillus* product supplementation on calf performance (Yohe et al., 2015, Vaz-Ramírez et al., 2021), highlighting inconsistencies in responses that have hindered the widescale

industry application of these types of dietary interventions. Supplementing dairy calves with the mannan-rich fraction of *Saccharomyces cerevisiae* cell walls (MRF) (Alltech Inc., Kentucky, US), has been found to be beneficial for calf immunity by interacting with macrophages and neutrophils and engulfing pathogens in the GI tract, as well as enhancing gut development and epithelial integrity (Broadway et al., 2015, Ma et al., 2020). To our knowledge, there is no published research examining the effects of supplementing calves with ANP and MRF in combination. We hypothesized that combining the digestibility-enhancing potential of ANP with the immunomodulating capacity of MRF, could lead to additive or synergistic effects in calves by increasing nutrient availability and the capacity of the calves to utilise those nutrients through improved gut health. The objectives of this study were to measure the effects of ANP, alone, or in combination with MRF on calf growth, health, feed intake, FCE, digestibility, VFA production and rumen development.

6.3 Materials and Methods

6.3.1 Treatments, animals and management

All procedures in this study were approved by The University of Glasgow Research Ethics Committee (ref EA07/24). To determine minimum sample requirements, power analyses were conducted for intake, ADG, and rumen variables ($\alpha = 0.05$, $\beta = 0.1$, Power = 0.8). Thirty Holstein-Friesian bull calves, age 19 ± 5 d, weight 52 kg (SD = 18), were sourced from a single farm and transported to Cochno Farm and Research Centre (Clydebank, UK) for this 70-d trial. Upon arrival, calves were blocked into six groups age groups, and then randomly assigned to a cull date, trial day 27 or 70, and to evenly balanced dietary treatment groups according to age. Groups were: CTRL, no treatment; ANP, 3 g/d ANP; MRF, 3 g/d ANP and 2 g/d MRF; single calf was the experimental unit in this study. For the first seven days of the trial, calves were adapted to treatments and environment; no data collected in the adaptation period were used in analyses. On day three of the adaption period, calves were

vaccinated intranasally against pneumonia (Rispoval RS + PI3, Zoetis, NJ). Calves were housed in a Yorkshire board barn (solid, cement block to 1.75 m height, with vertical timber slats, spaced at 50 mm intervals to 2.65 m height) and individually penned. In the pre-weaned period, pens measured 1.8 × 1.8 m and post-weaning they were expanded to 1.8 × 3.6 m. Pens were bedded with barley straw, which was top-filled daily and mucked out every two weeks. Calves had *ad libitum* access to fresh water, starter concentrates (I'Anson Early Weaner Pellets, 18 % CP, 8.75 % CF, 4.25 % fat, 7.5 % ash) and barley straw for the duration of the trial. For the first 27 d, calves were fed 6 l of whey protein-based milk replacer (MR) (Volac Heiferlac, 26 % CP, fat 16 % fat, 19.7 Mj ME/kg dry matter (DM)), split into two equal feeds offered at 0700-h and 1600-h. MR was offered at 38 – 40 °C at a concentration of 150 g DM milk power diluted in 0.85 l water. In the pre-weaned period, calves received treatments in the morning MR feed, which was administered using 3 l bottles. Bottles were agitated manually during feeding to keep treatments in suspension; the afternoon feed was offered in Single Teat Calf Feeders (Stockman, Belfast, UK). Milk replacer consumption was monitored at every feed and no milk refusals were observed during the trial. On day-27, all calves received MR in the morning and calves assigned to the day-70 cull were abruptly weaned. Pre-weaning, concentrate refusals were measured daily whereby calves were provided with a known quantity of concentrates in the morning and refusals were weighed using a digital balance (OHAUS, Lecesiter, UK), 24 h later. In the weaned period, the top layer of concentrates in each calf's morning feed, was sprayed with water before treatments were dusted on top and the bucket gently agitated to adhere treatments to pellets. Calves were provided with a quantity of concentrates anticipated to be lower than their daily consumption to ensure complete consumption of treatments, once all the treated concentrates had been consumed, more was offered. This approach was applied to all animals; both quantities of initial and top-up concentrates were recorded and concentrates were weighed prior to being sprayed with water to ensure accurate DM intake calculations. The quantities offered were adjusted daily based upon consumption the previous day and no upper limit was placed upon the quantity

of concentrates a calf could consume, quantities of starter offered, refused, and consumed are recorded in the online supplementary file associated this paper (https://doi.org/10.5525/gla.researchdata.2018). Samples of concentrates, straw and MR were taken weekly and pooled every four weeks for nutrient analysis.

6.3.2 Growth, digestibility and health measurements

In the pre-weaned period, calves were weighed on two consecutive days weekly and, in the weaned period, calves were weighed on two consecutive days every other week. Weight measurements were recorded using a calf weight crate with digital scales (Ritchie Agricultural, Angus, UK), immediately after the morning feed on both days, and the mean of the two consecutive weights was used in FCE and average daily gain (ADG) calculations. Average daily gain was calculated by dividing weight gain by the number of days since weight was last measured. FCE was calculated by dividing ADG by the daily DM intake of starter and MR. Faecal health was measured on two consecutive days weekly, using the Wisconsin Madison scoring system, where 0 = normal; 1 = semi-formed and pasty, 2 = loose but stays on top of bedding, 3 = watery and sifts through bedding (McGuirk, 2008). On alternate weeks from week one of the trial, spot samples of ≈ 20 g faeces were collected after faecal scoring and immediately frozen at - 20 °C. Following the trial, apparent total tract digestibility of DM was estimated using the acid insoluble ash (AIA) protocol of Van Keulen and Young (1977) Calculations were based on the AIA content of MR and concentrates that each calf had consumed 24 h prior to faecal collection. Since calves were bedded on barley straw, as well as having *ad lib* access to straw in feeders, the level of straw intake could not be accurately quantified, as such, the AIA content of straw was not included in calculations, highlighting a potential limitation of this dataset.

6.3.3 Rumen variables

On days 27 and 70, five calves from each treatment group were culled using Schedule 1 Killing, via administration of intravenous pentobarbital at a dose of 0.7 ml/kg liveweight. After confirmation of permanent cessation of circulation was verified by auscultation for heart sounds, the rumen of each calf was removed. Duplicate 10 ml samples of digesta were collected from the ventral sac of rumens and stored with 0.5 ml phosphoric acid at -80 °C prior to VFA analysis using gas chromatography. The concentrations of total VFA (tVFA), acetate, propionate, butyrate, valerate, isobutyrate and isovalerate in centrifuged rumen digesta were quantified using a Vapor Fractometer and column containing 2 % phosphoric acid and 20 % Tween-80 (Perkin-Elmer, Waltham, Massachusetts, US), following the protocol of (Erwin et al., 1961). After digesta collection, rumens were emptied, rinsed with cold phosphate-buffered saline and weighed. A 3×3 cm section of the ventral and dorsal sacs was removed, pinned onto cardboard and fixed in 10 % formalin for 48 h. To ensure homogeneity of sampling location, the same operator collected tissue samples from all rumens. After being fixed in formalin, a slice (3 µm thickness) of each ventral and dorsal sac sample was embedded in paraffin slides, which were then stained with haematoxylin and eosin (H&E) and scanned at ×40 magnification. Staining and scanning were performed by Veterinary Diagnostic Services at The University of Glasgow. Rumen papillae were measured using QuPath-0.5.1 (Bankhead et al., 2017) and treatment identifiers were masked during analyses. The five longest papillae on each slide were selected for analysis; the length of each papilla was measured using a straight line from base to tip. If papillae were sufficiently bowed to prevent a single line that passes through the body of a papilla being drawn, a polyline was used. Papilla width was estimated by taking the mean of five straight lines drawn from the ventral to dorsal plane of each papilla. The first width measurement was always taken at the transection point of the papilla and lines were drawn at approximately even intervals thereafter, whilst avoiding crossing the papilla at branches, which would disproportionately skew the mean. The thickness of the stratum corneum (SC) and combined thickness of the strata

basale, spinosum and granulosum (SBSG) were estimated by taking the mean of five measurements from each papilla. Lines for SC and SBSG were drawn perpendicular to the junction between the SC and the stratum granulosum (Fig. 6-1). The ratio of keratinized to non-keratinized epithelium of each papilla was estimated by dividing the SC thickness by the SBSG thickness at each measurement point and taking the mean of those five measurements. A slide from the ventral sac of one calf (calf 319, ANP, week 13) was excluded from analysis due to mechanical damage that prevented accurate papillae assessment.

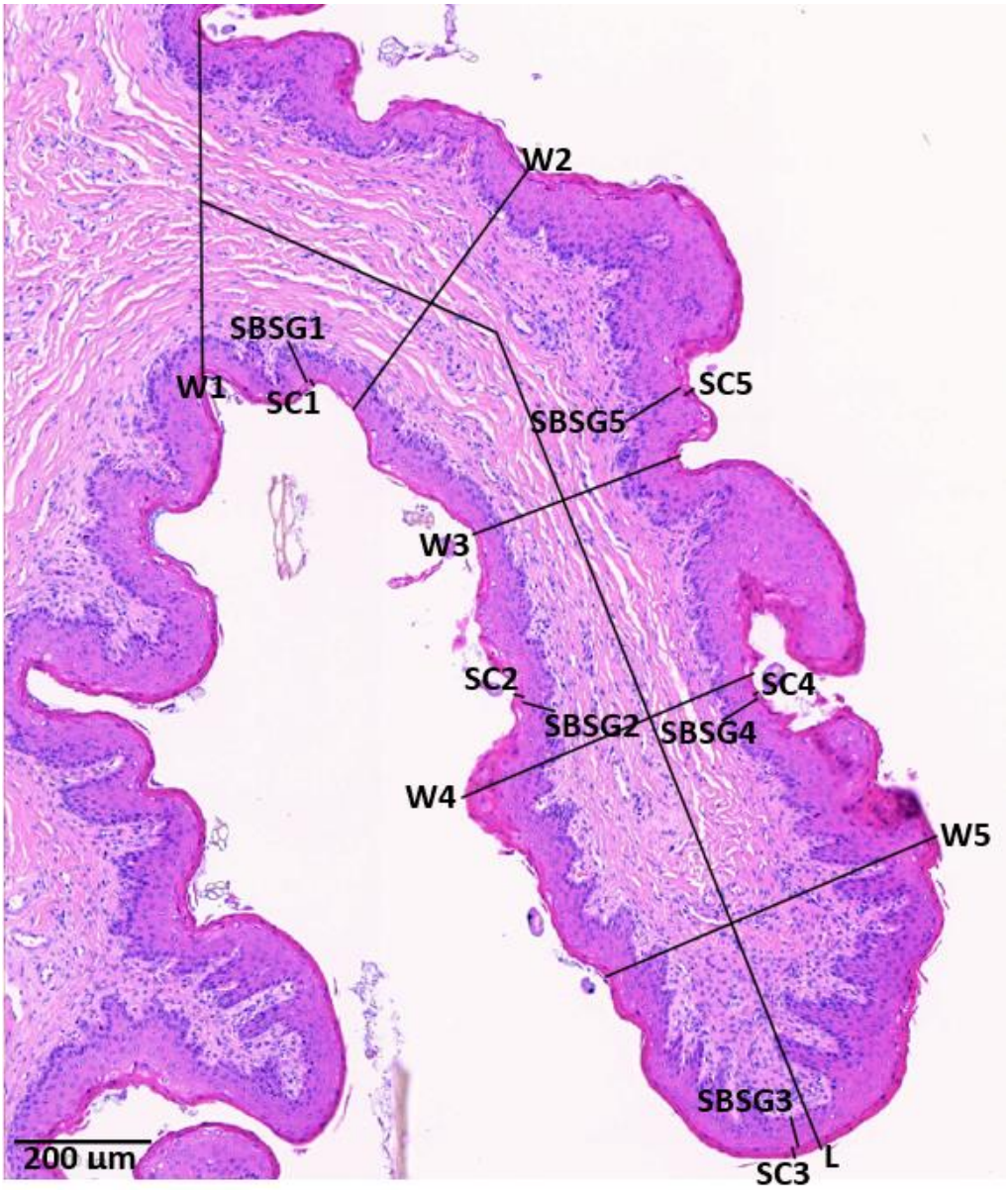


Figure 6-1 Measurements used to quantify rumen papillae morphology in pre-weaned and weaned dairy calves. Papillae width = average W1: W5, W1 = transection point of papilla. Papillae length (L) = straight line from W1 - tip, or polyline if a straight line that passes through the papilla cannot be drawn due to bowing. Stratum corneum (SC) thickness = average SC1: SC5. Strata basale, spinosum, granulosum (SBSG) thickness = average SBSG1: SBSG5. Lines for SC and SBSG were drawn perpendicular to the junction between SC and stratum granulosum.

6.3.4 Statistical analysis

Feed intake, FCE, ADG and apparent total tract DM digestibility data were analysed using repeated measures (RM) ANOVA with treatment and time as factors. Papillae length, width, SC thickness, SBSG thickness and SC: SBSG data were analysed using RM ANOVA with treatment and calf as factors. Histological data from each cull timepoint and ventral and dorsal sacs were analysed in separate models. Datasets were subjected to Shapiro Wilks normality tests, if the assumptions of the tests were not met ($P < 0.05$), then the data were transformed using $\log_{10}$ or $1/x$ transformations; rumen weight, VFA concentrations and proportions and body weight (BW) data were analysed using one-way ANOVA. When ANOVA indicated significant differences between groups due to treatment effects, Tukey's Honestly Significant Difference *post hoc* test was used to assess differences between means and significance was declared at $P < 0.05$. The majority of rumen histology data were non-parametric; data characteristics for each rumen variable, and the transformation used to meet normality assumptions, are summarized in Table 6-1. Faecal score data were non-parametric, and no attempted transformation met normality assumptions, so differences were analysed using a Kruskall Wallis H test. All analyses were performed in R (R Core Team, 2024), except for faecal data analysis which was performed using a statistical process control add-in for Microsoft Excel (QI Macros 2025, KnowWare International).

Table 6-1 Characteristics of calf rumen histology data and transformations used to meet normality assumptions where data were non-parametric.

Timepoint	Tissue	Parameter	Distribution	Transformation
Week-7	Ventral sac	Pap length	Non-parametric	Log10
		Pap width	Non-parametric	1/X
		SC thickness	Non-parametric	Log10
		SBSG thickness	Normal	-
		SC: SBSG	Non-parametric	Log10
	Dorsal sac	Pap length	Non-parametric	Log10
		Pap width	Non-parametric	Log10
		SC thickness	Non-parametric	Log10
		SBSG thickness	Non-parametric	Log10
		SC: SBSG	Non-parametric	1/X
Week-13	Ventral sac	Pap length	Non-parametric	Log10
		Pap width	Non-parametric	Log10
		SC thickness	Non-parametric	Log10
		SBSG thickness	Non-parametric	1/X
		SC: SBSG	Non-parametric	Log10
	Dorsal sac	Pap length	Normal	-
		Pap width	Normal	-
		SC thickness	Non-parametric	1/X
		SBSG thickness	Non-parametric	1/X
		SC: SBSG	Non-parametric	1/X

Abbreviations: SC = stratum corneum; SBSG = strata basale, spinosum and granulosum; pap = papillae.

6.4 Results

All calves remained healthy throughout this trial, no milk refusals were observed in the pre-weaned period, and no cases of morbidity were observed throughout. Mean BW of calves was 52 kg (SD = 18) at the beginning of the trial, 73 kg (SD = 10) at weaning, and 122 kg (SD = 20) at the end of the trial. ADG in the pre-weaned period was 789 g/d (SD = 43) and 1177 g/d (SD = 47) in the weaned period.

6.4.1 Intake, growth and faecal variables

Results for primary response variables: FCE, ADG, starter intake and BW are shown in Table 6-1. Body weight and ADG were similar among all treatments in pre-weaned and weaned periods. ANP calves consistently consumed more starter concentrates throughout the trial. In the pre-weaned period, ANP calves had a mean intake of 417 g/d, higher than the CTRL calf intake of 360 g/d (P = 0.044) and MRF calf intake of 328 g/d (P = 0.0006). In the weaned period, ANP calves had a mean starter intake of 3398 g/d, which was significantly higher than the CTRL intake of 3155 g/d (P = 0.021) and MRF intake of 3203 g/d (P =0.0027). Time had a significant effect on intake across both periods (P < 0.0001) and there was no interaction between time and treatment on pre-weaned intake (P = 0.97) or weaned intake (P = 0.051). Treatments did not consistently affect FCE in the pre-weaned or weaned period although some differences between groups in weekly and bi-weekly data were apparent (Table 6-2). Time significantly affected FCE in the pre-weaned and weaned periods (P < 0.0001) and there was an interaction between time and treatment in the pre-weaned period (P = 0.024). Faecal score was unaffected by treatment in the pre-weaned (P = 0.82) and weaned (P = 0.14) periods; mean faecal scores for all groups in both periods was < 1. The apparent total tract digestibility of DM was estimated to be 72.2 % in CTRL, 70.5 % in ANP and 71.5 % in MRF and was unaffected by treatment (P = 0.52), time (P = 0.32) and there was no interaction between treatment and time (P = 0.12).

Table 6-2 Feed conversion efficiency, starter concentrate intake, average daily gain and bodyweight of Holstein Friesian bull calves fed 3 g/d Aspergillus niger fermentation extract alone (ANP), or in combination with 2 g/d of the mannan rich fraction of Saccharomyces cerevisiae cell wall (MRF), compared with control calves offered no treatments (CTRL).

Variable	Treatment			Pooled SE	Probabilities		
	CTRL	ANP	MRF		Treatment	Time	Treatment × Time
BW (kg)							
Arrival (n=30)	51.6	52.7	52.4	1.72	0.97	-	-
At weaning (d-27) (n=30)	71.8	75.2	73.4	1.88	0.77	-	-
Final (d-70) (n=15)	117.7	130.2	119.6	5.47	0.64	-	-
ADG (g)							
Pre-weaned (n=30)	773	817	778	7.9	0.63	< 0.01	0.77
Weaned (n=15)	1110	1236	1184	12.05	0.47	< 0.01	0.73
Daily starter intake (g)							
Pre-weaned (n=30)	360[a]	417[b]	328[a]	11.52	< 0.01	< 0.01	0.97
Weaned (n=15)	3155[a]	3398[b]	3203[a]	50.17	< 0.01	< 0.01	0.05
FCE							
d-13	0.64[a]	0.64[a]	0.69[b]	0.008	0.02	0.37	0.93
d-20	0.62	0.62	0.63	0.009	0.94	0.78	0.94
d-27	0.63[a]	0.59[b]	0.60[a,b]	0.007	0.03	0.02	0.32
Entire pre-weaned period (n=30)	0.63	0.62	0.64	0.006	0.21	< 0.01	0.02
d-42	0.56	0.55	0.61	0.025	0.45	< 0.01	0.70
d-56	0.34[a]	0.38[b]	0.34[a]	0.007	< 0.01	< 0.01	0.16
d-70	0.30[a]	0.32[a,b]	0.33[b]	0.003	< 0.01	< 0.01	0.20

Entire weaned period (n=15)	0.40	0.42	0.43	0.001	0.44	< 0.01	0.58

Abbreviations: FCE = feed conversion efficiency; ADG = average daily gain; BW = bodyweight.

[a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

6.4.2 Rumen variables

The molar concentrations and proportions of VFA in centrifuged rumen digesta collected at weeks seven and 13 are shown in Table 6-3. In week-7, the acetate: propionate ratio (Ace: Prop) in MRF was 2.18, which was significantly lower than the Ace: Prop of 2.7 in CTRL ($P = 0.047$). This is likely caused by a tendency for MRF to have lower acetate % than CTRL (55 vs. 60 %) ($P = 0.071$) and a higher propionate % than CTRL (26 vs. 23 %) ($P = 0.059$). There was no effect on any other VFA variable in week-7. In week-13, ANP calves had an Ace: Prop of 1.6, significantly lower than the 2.27 A: P of CTRL ($P = 0.0085$) and a significantly higher propionate % than CTRL (32 vs. 25 %, $P = 0.004$). Differences in valerate concentrations and isoacid concentrations and proportions were also observed between groups in week-13. The variables derived from rumen tissue are presented in Table 6-4. ANP calves had consistently wider ruminal papillae, in ventral and dorsal sacs, at 7 and 13 weeks. Both ANP and MRF calves had higher SGSB thickness than control calves, in both locations and at both timepoints.

Table 6-3 Volatile fatty acid concentrations and proportions in rumen digesta collected from Holstein Friesian bull calves fed 3 g/d Aspergillus niger fermentation extract alone (ANP), or in combination with 2 g/d of the mannan-rich fraction of Saccharomyces *cerevisiae* cell wall (MRF), compared with control calves offered no treatments (CTRL).

		Treatment				
Trial day	Variable	CTRL	ANP	MRF	Pooled SE	*P*-value
27	tVFA (mM)	115.50	135.50	127.15	5.27	0.28
	Acetate (mM)	68.93	79.22	70.52	3.42	0.65
	Propionate(mM)	26.23	31.27	33.11	1.28	0.07
	Butyrate (mM)	14.91	19.19	18.06	0.978	0.18
	Valerate (mM)	2.46	3.50	3.30	0.268	0.24
	Isovalerate (mM)	1.88	1.45	1.31	0.151	0.76
	Isobutyrate (mM)	1.10	0.867	0.854	0.0642	0.22
	Acetate: propionate	2.70[a]	2.51[a,b]	2.18[b]	0.0904	0.06
	Acetate (% tVFA)	60.06	57.98	55.28	0.893	0.09
	Propionate (% tVFA)	22.71	23.30	26.32	0.662	0.062
	Butyrate (% tVFA)	12.66	14.28	14.12	0.457	0.29
	Valerate (% tVFA)	2.07	2.51	2.57	0.149	0.34
	Isovalerate (% tVFA)	1.56	1.21	1.04	0.118	0.20
	Isobutyrate (% tVFA)	0.95	0.72	0.67	0.0513	0.07

70	tVFA (mM)	168.69	168.21	156.67	4.92	0.54
	Acetate (mM)	92.42	84.42	81.26	3.33	0.38
	Propionate(mM)	41.75	53.91	46.01	2.31	0.06
	Butyrate (mM)	24.36	21.07	20.81	0.824	0.15
	Valerate (mM)	5.41[a]	7.62[b]	7.17[a,b]	0.339	0.02
	Isovalerate (mM)	3.565[a]	0.772[b]	0.92[b]	0.271	< 0.01
	Isobutyrate (mM)	1.18[a]	0.423[b]	0.496[b]	0.0928	0.01
	Acetate: propionate	2.27[a]	1.60[b]	1.85[a,b]	0.103	0.01
	Acetate (% tVFA)	55.40	50.12	51.66	0.898	0.14
	Propionate (% tVFA)	24.94[a]	32.01[b]	29.12[a,b]	0.951	< 0.01
	Butyrate (% tVFA)	14.61	12.61	13.70	0.563	0.36
	Valerate (% tVFA)	3.28[a]	4.52[b]	4.54[b]	0.170	< 0.01
	Isovalerate (% tVFA)	2.07[a]	0.48[b]	0.64[b]	0.148	< 0.01
	Isobutyrate (% tVFA)	0.69[a]	0.25[b]	0.35[b]	0.0550	< 0.01

Abbreviations: tVFA = total volatile fatty acids. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

Table 6-4 Rumen weights and papillae variables in Holstein Friesian bull calves fed 3 g/d Aspergillus niger fermentation extract alone (ANP), or in combination with 2 g/d of the mannan rich fraction of Saccharomyces cerevisiae cell wall (MRF), compared with control calves offered no treatments (CTRL).

			Treatment				Probabilities		
Trial day	Tissue	Parameter	CTRL	ANP	MRF	Pooled SE	Treatment	Animal	Treatment × Animal
27	Ventral sac	Pap length (µm)	1567	1780	1806	81.5	0.40	0.01	0.53
		Pap width (µm)	316[a]	345[a,b]	409[b]	16.2	0.03	0.37	0.68
		SC thickness (µm)	12.83[a]	9.45[b]	12.03[a]	0.435	< 0.01	0.10	0.29
		SBSG thickness (µm)	61.89[a]	76.08[b]	68.91[a,b]	1.73	< 0.01	0.05	0.37
		SC: SBSG	0.245[a]	0.141[b]	0.199[a]	0.0250	< 0.01	0.63	0.22
	Dorsal sac	Pap length (µm)	1171[a]	1530[b]	1158[a]	68.5	< 0.01	< 0.01	< 0.01
		Pap width (µm)	379[a]	404[a,b]	494[b]	20.0	0.03	0.13	0.26
		SC thickness (µm)	12.07	10.26	11.26	0.410	0.12	0.01	0.03

		SBSG thickness (µm)	55.66[a]	71.36[b]	76.05[b]	1.79	< 0.01	0.04	0.34
		SC: SBSG	0.244[a]	0.194[b]	0.162[b]	0.0350	< 0.01	0.67	0.02
	Rumen weight (g)		1000	1011	1048	40.7	0.90	-	-
70	Ventral sac	Pap length (µm)	3754[a]	2325[b]	4116[a]	232	< 0.01	0.24	0.01
		Pap width (µm)	329[a]	427[b]	334[a,b]	14.8	0.01	0.05	0.29
		SC thickness (µm)	14.58	14.37	15.02	0.565	0.81	< 0.01	0.44
		SBSG thickness (µm)	63.37[a]	84.65[b]	81.92[b]	2.84	< 0.01	0.17	< 0.01
		SC: SBSG	0.255	0.207	0.204	0.0097	0.05	< 0.01	0.16
	Dorsal sac	Pap length (µm)	2867[a]	4164[b]	4134[b]	164	< 0.01	0.82	0.80
		Pap width (µm)	373[a]	471[b]	431[a,b]	12.8	0.01	0.85	0.31
		SC thickness (µm)	16.94[a]	19.65[b]	17.96[a]	0.996	< 0.01	0.05	0.29
		SBSG thickness (µm)	68.57[a]	85.33[b]	92.4[b]	2.46	< 0.01	0.02	0.09

SC: SBSG	0.283[a]	0.272[a]	0.232[b]	0.0170	< 0.01	< 0.01	0.28
Rumen weight (g)	3366	3574	3511	153	0.87	-	-

Abbreviations: pap = papilla; SC = stratum corneum; SBSG = strata basale, spinosum and granulosum. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

6.5 Discussion

Supplementing dairy calves with ANP alone or in combination with MRF, did not consistently improve FCE or ADG, although ANP calves had consistently greater starter intake than both CTRL and MRF throughout the trial. There was a shift in rumen fermentation patterns towards propionate in both treatment groups, but at different stages of calf development, MRF pre-weaning and ANP post-weaning. Isoacids were also decreased and valerate was increased in MRF and ANP calves post-weaning. The layer of metabolically active cells (SBSG) in ANP papillae was thicker than CTRL at both culling timepoints, in both ventral and dorsal sacs. This effect was also measured in MRF apart from in the ventral sac at week seven. Increases in papillae dimensions were measured in both MRF and ANP compared with CTRL, although these effects were not consistent across tissue sampling sites and culls; no additive or synergistic effects between MRF and ANP were seen for any variable.

The dominant proposed mode of action of *Aspergillus* fermentation products in ruminants is prebiotic, whereby feed digestibility can be increased, either through residual enzyme activity in the products, and/or the nutritional content of the product can act as a substrate to promote favourable microbial population growth in the rumen (Uwineza et al., 2024). In the present study, ANP did not enhance calf growth or consistently improve the conversion of concentrates into BW. These results are in line with the study of Vaz-Ramírez et al. (2021), which measured no effect of *A. niger* and *A. oryzae* fermentation extract supplementation (2 g/d) on Jersey and Holstein calf growth and skeletal variables. Our study measured a significant increase in concentrate intake in calves fed ANP; increased intake, without corresponding increases in ADG or FCE, would likely be considered economically undesirable in more mature dairy or beef animals. However, given the positive impact that concentrate intake has on rumen development and the transition from milk to solid-feed in calves (Van Amburgh et al., 2014), we suggest that in the timeframe of this study, the increased concentrate intake measured in ANP is a beneficial outcome given the improvements measured in rumen variables. Di Francia et al. (2008) supplemented buffalo calves with *A. oryzae* and *S. cerevisiae* cultures at 6 g/kg

and 26g/kg DM intake, respectively, and reported similar starter intakes across treatment and control groups. However, the digestibility of DM, OM, CP and NDF was increased in calves fed cultures. As in the present study, Di Francia et al. (2008) used AIA as an internal marker to estimate digestibility, but they quantified the amount of forage calves consumed. Not measuring straw intake is a limitation of the digestibility data produced in this study; accurate quantification of straw intake was impractical due to a condition of the ethics license for this study being that calves must be bedded on straw. If treatments affected forage intake or fibre digestibility, differences between groups may have been undetected by not including straw AIA in analyses. The faecal health of calves in this study was unaffected by treatment. We did not anticipate an effect of ANP on faecal scores, however, MRF supplementation has previously improved the faecal health of dairy calves (Heinrichs et al., 2013, Spring et al., 2015). The mode of action of MRF is suggested to be prebiotic, where supplementation can promote gut integrity via the binding, and limiting colonization of, gut pathogens, including *Escherichia coli* and *Clostridia* spp., resulting in improved gut health and decreased diarrhoea incidence (Spring et al., 2015). In this study, calves remained healthy throughout, demonstrated by a mean faecal score of < 1 in all groups. Any benefits of MRF are likely to be most apparent in challenged environments, as such, conclusions on the effect of MRF on calf health should not be drawn from these data. Research performed in less hygienic conditions, where treatments can be administered earlier in life than the present study, might enhance the understanding of MRF effects when calves encounter disease stressors.

Apart from a lower Ace: Prop measured in MRF calves; rumen fermentation patterns were similar between groups pre-weaning. Decreased Ace: Prop and a tendency for increased propionate % in MRF is indicative of an increase in the more efficient propionate fermentation pathway. Butyrate and propionate are considered the most important VFA for rumen development in calves, providing a source of energy for the proliferation and growth of papillae (Diao et al., 2019). In our study, calves in all treatment groups had similar butyrate concentrations, in line with the results of Beharka et al. (1991), who saw no differences in butyrate between controls and calves fed *A. oryzae* fermentation

product. However, they noted an increase in acetate and propionate concentrations in treatment calves. The changes in fermentation pattern in MRF could have provided more energy to the rumen epithelium, leading to wider papillae, a thicker layer of metabolically active cells (SBSG) and a lower ratio of keratinized (SC) to non-keratinized (SBSG) cells, compared with CTRL, at week seven. Although ANP did not have a significantly different VFA profile to CTRL pre-weaning, we also measured improved papillae morphology in their rumens; thicker SBSG and lower SC: SBSG in both ventral and dorsal sacs, and longer papillae in the ventral sac, compared with CTRL. This is converse to the study of Yohe et al. (2015), which found no difference in papillae length and width in Holstein bull calves treated with or without 2 g/d *A. oryzae* fermentation extract. The differences we measured could be associated with the increased concentrate intake measured in ANP, which would have increased their dietary energy intake. Shen et al. (2004) proposes that VFA concentrations are not the sole driver of papillae development in young ruminants and that increased intake of metabolizable energy increases the concentration of insulin-like growth factor (IGF)-1 in the rumen and IGF-1 receptors in papillae. Thus providing a functional mechanism for papillae to metabolize the increased nutrients provided by increased concentrate intake, resulting in morphological enhancements in the developing rumen (Shen et al., 2004). The effect of SC thickness on rumen function in calves is not well defined. Keratinocytes provide a barrier against pathogenesis, increase the surface area of the rumen for digestion, and play an active and passive role in the transport of nutrients to the rumen mucosa (Baaske et al., 2020). However, these cells do not catabolize VFA and we suggest that lower SC: SBSG measured in ANP and MRF is advantageous because it indicates a greater proportion of metabolically active cells in papillae. Notably, in the pre-weaned period, treatments were administered in milk so may not have interacted directly with rumen tissue and microbes having bypassed the reticulorumen through the oesophageal groove. Given the beneficial effects on rumen histology that were measured in treatment calves, investigation into the effects of ANP and MRF throughout the rest of the GI tract would be beneficial.

At week 13, ANP calves had increased propionate % and decreased Ace: Prop compared with CTRL calves. The propionate pathway is more efficient than

acetogenesis (Janssen, 2010), a shift towards propionate could indicate enhanced starch hydrolysis and/or increased propionate could be associated with the increased concentrate intake, leading to enhanced papillae morphology measured in ANP. Post-weaning MRF and ANP had increased valerate proportions compared with CTRL. Kristensen and Harmon (2004) suggest that butyrate and valerate compete for the same metabolic pathway in rumen epithelium, as such, increased valerate metabolism could affect the absorption of butyrate. VFA metabolism and absorption rates cannot be quantified from a single VFA measurement, however, if butyrate absorption was limited by increased valerate in ANP and MRF, it did not negatively affect the rumen variables that we measured. In non-ruminants, increased valerate has been associated with improved intestinal barrier function (Gao et al., 2022) and increased FCE (Zhang et al., 2020), further research is required to investigate the effects of varying valerate concentrations in calves. In MRF and ANP, concentrations and proportions of isovalerate and isobutyrate were significantly decreased, compared with CTRL at week 13. This effect was also measured by Beharka et al. (1991) in Holstein heifer calves supplemented with *A. oryzae* extract. Isoacids come from the de-amination of proteins and are important substrates for cellulolytic bacteria growth (Andries et al., 1987); Beharka et al. (1991) suggests that decreased isoacid concentrations could indicate increased isoacid assimilation by cellulolytic bacteria. Supplementing calves with exogenous isoacids has improved calf growth, feed efficiency and ruminal cellulolytic bacteria counts (Liu et al., 2016), and feed intake, papillae length and width (Wang et al., 2017). However, 'normal' endogenous levels of isoacids in calves are undefined, so it is difficult to determine if the concentrations of isoacids in MRF and ANP would have affected cellulolytic bacteria proliferation without microbiome data. The effect of ANP and MRF on rumen microbial dynamics requires further investigation as this could provide evidence that explains the changes in fermentation patterns measured in this study. At week 13, ANP calves had shorter and wider papillae than CTRL in the ventral sac, and longer and wider papillae than CTRL in the dorsal sac; MRF had longer papillae than CTRL in the dorsal sac. This indicates that treatments could increase the surface area of papillae, although this was not consistent over the whole rumen. Similarly to

week seven, MRF and ANP had thicker layers of SBSG in both ventral and dorsal sacs. Cells in the SBSG catabolize VFA through ketogenesis, providing energy for the growth and maintenance of the rumen mucosa, and the entire animal (Baaske et al., 2020). SBSG thickness is particularly important post-weaning, where the metabolic capacity of papillae is fundamentally linked to calf performance, with VFA providing up to 70 % of ruminant energy requirements (Bergman, 1990). As such, increases in gross papillae structure and the thickness of the SBSG suggest increased capacity to perform VFA metabolism.

6.6 Conclusion

The changes in papillae morphology we measured in calves supplemented with ANP and ANP + MRF indicate that treatments could enhance the metabolic and absorptive capacity of the rumen in transition stage calves. These differences would be theoretically consistent with improved efficiency, but there was no evidence of a direct effect of treatment on growth and FCE metrics in this small study. Data from commercial trials using a larger number of calves, in more challenged conditions, are required to draw conclusions about the effects of treatments on calf performance.

Chapter 7. Comparison of calf faeces and rumen digesta as inoculum to measure *in vitro* digestibility using the gas production technique

Bridge to Chapter 7

Access to the rumen contents of the calves used in the previous study, as well as five, 4-wk old calves from the same farm, enable an additional *in vitro* digestibility experiment to be performed. As highlighted in both reviews in this thesis, *in vitro* digestibility models are useful tools to test the efficacy of dietary interventions in ruminants. However, it is not usually acceptable to fistulate calves, so obtaining rumen digesta to use as microbial inoculum for *in vitro* experimentation is more challenging. Chapter 7 describes an experiment which compared the gas production kinetics from incubations using faeces and rumen digesta as microbial inocula. This experiment aimed to test if faeces could be a useful proxy for rumen digesta in calf digestibility models.

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7.1 Abstract

This Research Communication addresses the hypothesis that calf faeces can be used as a microbial inoculum in the *in vitro* gas production technique to model rumen digestibility in calves. *In vitro* gas production is a valuable method of screening dietary interventions in ruminants. However, the technique requires a microbial inoculum, typically obtained from the rumens of fistulated animals. Using faecal inocula that can be collected non-invasively would be ethically and economically preferable, if it provided equivalent results. The aim of this pilot study was to compare *in vitro* gas production from faecal and ruminal inocula

obtained from dairy calves that were killed either pre-weaning or post-weaning. Ruminal content and faeces were collected from calves aged 4, 7 and 13 weeks old, and incubated with calf starter concentrates in the ANKOM^{RF} system. A weak relationship between inocula was seen for all modelled gas production kinetic parameters (R^2 < 0.53); although modelling indicated that incubations using faecal and rumen inocula from pre-weaned calves had similar lag times and rates of degradation. In weaned calves, aged 13 weeks, faecal incubations produced less gas, lag times were longer, and the rate of degradation of the slowly degradable fraction of calf starter was slower, compared to rumen incubations. Weak relationships, and kinetic differences, between rumen and faecal inocula would likely hinder the use of faeces to model calf rumen fermentation.

Keywords*:* dairy calves; *in vitro* digestibility; gas production; faecal modelling; pre-ruminant

7.2 Introduction

In cattle, measuring *in vitro* digestibility using the gas production (GP) technique is valuable because it facilitates the screening of large numbers of dietary interventions using rumen inoculum derived from a small number of animals. However, the collection of rumen contents is invasive, usually requiring fistulated animals, making the exploration of alternative inoculum sources ethically and economically valuable. The use of faeces in GP systems has been investigated in mature cattle, where faecal inocula were found to have a lower fermentative capacity and different rates of degradation compared to rumen liquor, in spite of strong correlations between inocula for GP: R^2 = 0.83 in grass incubations and R^2 = 0.94 in maize silage incubations, and OM digestibilities: R^2 = 0.81 in grass incubations and R^2 = 0.92 in maize silage incubations (Mauricio et al., 2001, Zicarelli et al., 2011). To our knowledge, the use of faecal inoculum to model digestibility in calves has not been investigated. Neonatal calves are pre-ruminants with underdeveloped reticulorumens and a digestive tract akin to monogastrics (Drackley, 2008), the microbial populations found throughout the calf digestive tract differ from, and are more dynamic, than those found in

mature cattle (Anderson et al., 1987). This means that results from *in vitro* GP experiments using rumen inoculum from mature cattle may not be translatable to calves and, as such, defining a method to accurately model digestibility in the calves is needed. Notably, the GP technique has been successfully adapted to use faecal inoculum from monogastrics, including pigs (Bindelle et al., 2007), and hindgut fermenters, such as horses (Lowman et al., 1999). Physiological similarities between monogastrics and pre-ruminants could mean that faeces has more utility in calf models compared with models measuring digestibility in mature cattle. The aim of this pilot study was to obtain a preliminary assessment of an *in vitro* system that uses faeces as a microbial inoculum to model digestibility in calves, by assessing and modelling GP profiles from faecal and rumen inocula from pre-weaned and weaned dairy calves.

7.3 Materials and methods

Twenty-five Holstein Friesian bull calves aged 19 ± 5 d, weight 52 kg (SD = 18), were obtained from a single farm. Calves were individually housed, given *ad libitum* access to barley straw, water and starter concentrates (I'Anson Early Weaner Pellets, 18 % CP, 8.75 % CF, 4.25 % fat, 7.5 % ash), and fed 6 l of milk replacer (Volac Heiferlac, 26 % CP, fat 16 % fat, 19.7 Mj ME/kg DM) split in two equal feeds in the pre-weaned period, before abrupt weaning at 48 ± 5 d-old. The ages, weights and starter intakes of calves at culls are displayed in the supplementary file associated with this paper which can be accessed through the DOI in the notes section of this communication. Upon arrival to the farm, calves were blocked by age and then randomly allocated to three balanced cull groups. Rumen and faecal digesta were collected at three timepoints, when five calves were culled at 27 ± 5 d-old (week 4), ten calves at 48 ± 5 d-old (week 7), and another ten at 90 ± 5 d-old (week 13). All animals remained healthy throughout the trial with no cases of morbidity or milk refusals observed. GP was performed using the ANKOM^{RF} system (ANKOM Technology, USA), adapted from Theodorou et al. (1994). Under a stream of CO_2, 10 ml of rumen digesta was collected from the ventral sac of each calf and 20 ml of faeces was collected rectally. Rumen

digesta and faeces were each combined with 50 ml of buffered mineral solution, the composition of which is described by Theodorou et al. (1994), and homogenised for 30 s using a blender. Solutions were strained through four layers of cheesecloth and 50 ml of the strained fluid was poured into 137 ml glass bottles. Each bottle contained 0.5 g calf starter, milled to pass through a 2 mm sieve and sealed in F57 fibre bags. The headspace of each bottle was flushed with CO_2 prior to the attachment of ANKOM^{RF} modules and bottles were placed in a water bath set to 39˚C, for 24 h. The timescale for incubations was decided due to starter (assumed to be rapidly digestible), rather than forage, being used as a substrate. This does limit the time available to observe effects of inocula on the slowly degradable fraction of the substrate, with typical *in vitro* incubations for mature cattle lasting 72 hours. Cumulative gas pressure from each module was measured automatically at 10 min intervals and, after incubations, F57 bags were removed from bottles, rinsed in de-ionised water and dried in a 55˚C oven overnight for the calculation of DM disappearance (DMD). Gas pressure was converted to moles using the Ideal Gas Law:

$$n = PV/RT$$

n = gas produced (mol)

P = pressure (kPA)

V = head-space volume in each bottle (l)

R = gas constant (8.314472L·kPa·K^{-1} mol^{-1})

T = temperature (K)

Moles were converted to ml using Avogadro's law where 1 mole of gas occupies a volume of 22.4 l at 273.15 ˚K and 101.325 kPa:

$$y = n \times 22.4 \times 1000$$

y = gas produced (ml)

n = gas produced (mols)

Gas production kinetics in ml were modelled using the SAS NLIN procedure (SAS University Edition, SAS Institute Inc., USA), to estimate parameters of the Cumulative Gas Production Equation of Schofield et al. (1994):

$$Y = (a/(1 + \exp(2 + 4 \times b \times (c - t)))) + (d/(1 + \exp(2 + 4 \times e \times (c - t))))$$

Y = cumulative gas produced (ml)

a = volume of gas produced from the rapidly degradable fraction (ml)

b = degradation rate of the rapidly degradable fraction (h^{-1})

c = lag time (h)

t = time (h)

d = volume of gas produced from the slowly degradable fraction (ml)

e = degradation rate of the slowly degradable fraction (h^{-1})

Data were subjected to Shapiro Wilks normality tests and found to be normally distributed. Comparison between estimates of each of the parameters of the Cumulative Gas Production Equation for rumen and faecal inocula of GP kinetics were performed using one-way ANOVA in R (R Core Team, 2024). The relationship between rumen and faecal GP kinetic parameters, total GP and DMD was estimated using linear regression in R:

$$lm(faecal \sim rumen, data = CalfGP)$$

7.4 Results and discussion

The GP profiles of rumen and faecal inocula at weeks 4, 7 and 13 are shown in Fig. 7-1, total GP and estimates for the parameters of the Cumulative Gas Production Equation of faecal inoculum on rumen inoculum GP kinetic parameters is shown in Table 7-1 and coefficients of determination between kinetic parameters from faecal and rumen incubations are shown in Table 7-2. In the pre-weaned incubation, faecal inoculum produced more gas than rumen inoculum, and in the incubations at weaning, and six weeks postweaning, rumen

inoculum produced a greater volume of gas that faecal inoculum. As shown in Fig. 7-1, there was large variation in GP between animals for both inocula, especially rumen inoculum at week-13, likely indicative of different levels of rumen development in the calves. Visually, Fig. 7-1 appears to show similar mean GP curves between faecal and rumen incubations, however, the results of linear modelling (Table 7-2), show a weak, or very weak, relationship between the GP kinetics of rumen and faecal incubations.

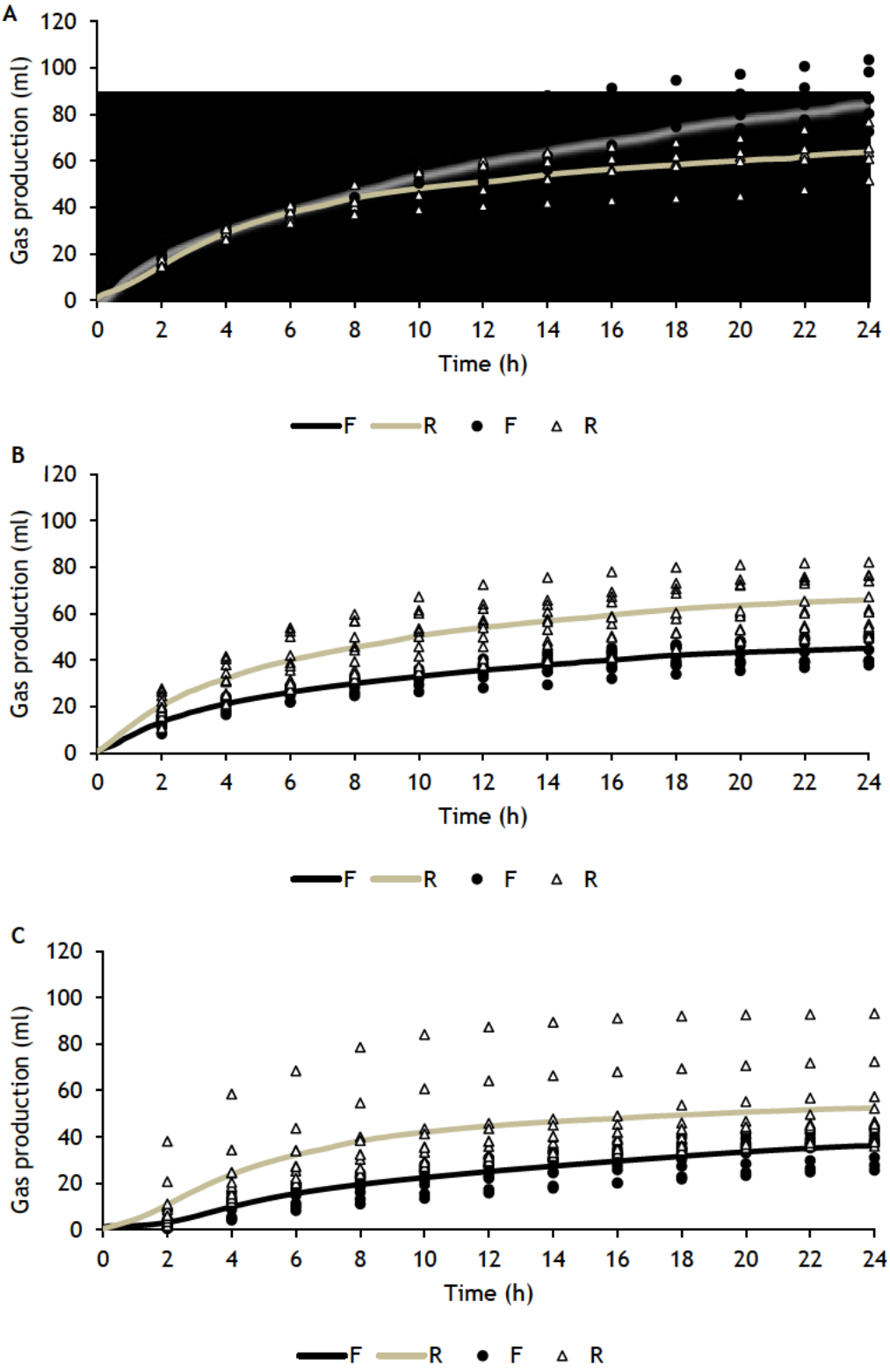


Figure 7-1 Gas production from 0.5 g calf starter incubated for 24-h with faecal and rumen inoculum obtained from Holstein Friesian bull calves. Solid lines show mean gas production measured at 10 min intervals using the ANKOM^{RF} GP system. Scatter points show GP from individual modules at 2 h intervals. A: Inoculum obtained from five 27 ± 5 d old calves. B: Inoculum obtained from ten 48 ± 5 d old calves. C: Inoculum obtained from ten 90 ± 5 d old calves.

Table 7-1 Estimates of gas production kinetics of 0.5 g calf starter incubated for 24 h, in the ANKOMRF system with faecal and rumen inoculum obtained from Holstein Friesian calves at 27, 48 and 90 days old.

		Inoculum			
Week	Parameter	Faecal	Rumen	Pooled SE	*P*-value
4	Total GP (ml)	88[a]	64[b]	5.24	< 0.01
	a (ml)	26	30	3.01	0.57
	b (h^{-1})	0.27	0.25	0.02	0.77
	c (h)	0.67	0.84	0.13	0.54
	d (ml)	67[a]	36[b]	6.05	< 0.01
	e (h^{-1})	0.05	0.05	0.005	0.62
	DMD (%)	51[a]	58[b]	1.49	< 0.01
7	Total GP (ml)	45[a]	66[b]	3.04	< 0.0001
	a (ml)	19[a]	27[b]	1.99	0.04
	b (h^{-1})	0.30	0.32	0.02	0.69
	c (h)	0.51	0.44	0.08	0.69
	d (ml)	29[a]	40[b]	1.86	< 0.01
	e (h^{-1})	0.05	0.06	0.003	0.17
	DMD (%)	47[a]	52[b]	0.90	< 0.001
13	Total GP (ml)	36[a]	52[b]	3.46	0.02

a (ml)	12[a]	23[b]	1.87	< 0.001
b (h^{-1})	0.30	0.31	0.01	0.87
c (h)	2.60[a]	1.50[b]	0.23	0.01
d (ml)	26	29	2.12	0.4963
e (h^{-1})	0.05[a]	0.07[b]	0.003	< 0.01
DMD (%)	55[a]	60[b]	0.77	< 0.0001

GP = gas production; a = gas derived from the rapidly degradable fraction of substrate; b = degradation rate of the rapidly degradable fraction; c = lag time of incubation; d = gas derived from the slowly degradable fraction of substrate; e = degradation rate of the slowly degradable fraction; DMD = dry matter disappearance. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

Table 7-2 Coefficients of determination between in vitro gas production kinetic parameters from faecal and rumen content incubations, obtained from Holstein Friesian calves at 27, 48 and 90 days old and incubated with 0.5 g calf starter for 24 h in the ANKOM^{RF} system.

		Inoculum				
Week	Parameter	Faecal	Rumen	Coefficient (R^2)	RSE	*P*-value
4	Total GP (ml)	88	64	0.138	9.69	0.54
	a (ml)	26	30	0.201	5.96	0.45
	b (h^{-1})	0.27	0.25	0.528	0.05	0.17
	c (h)	0.67	0.84	0.052	0.39	0.71
	d (ml)	67	36	0.459	5.51	0.21
	e (h^{-1})	0.049	0.054	0.006	0.02	0.90
	DMD (%)	51	58	0.114	1.63	0.58
7	Total GP (ml)	45	66	0.024	11.6	0.67
	a (ml)	19	27	0.005	10.7	0.84
	b (h^{-1})	0.30	0.32	0.150	0.09	0.27
	c (h)	0.51	0.44	0.024	0.34	0.67
	d (ml)	29	40	0.004	7.40	0.86
	e (h^{-1})	0.051	0.060	0.101	0.01	0.37
	DMD (%)	47	52	0.007	2.70	0.82
13	Total GP (ml)	36	52	0.150	17.6	0.27

a (ml)	12	23	0.134	8.07	0.30
b (h^{-1})	0.30	0.31	0.019	0.05	0.71
c (h)	2.6	1.5	0.008	0.86	0.81
d (ml)	26	29	0.404	10.3	0.05
e (h^{-1})	0.051	0.069[b]	0.086	0.02	0.41
DMD (%)	55	60	0.259	2.11	0.13

GP = gas production; a = gas derived from the rapidly degradable fraction of substrate; b = degradation rate of the rapidly degradable fraction; c = lag time of incubation; d = gas derived from the slowly degradable fraction of substrate; e = degradation rate of the slowly degradable fraction; DMD = dry matter disappearance; RSE = relative standard error. [a,b] Values not sharing a superscript are significantly different ($p < 0.05$).

In the pre-weaned incubation, faecal inoculum produced a significantly greater volume of total gas, with the increased gas predominantly arising from the slowly degradable of fraction of calf starter. There were no differences in the estimates for GP from the rapidly degradable fraction, or the fractional rates of degradation and lag time. This contrasts with studies using faeces from mature cattle, in which faecal inoculum produced less gas and incubations had increased lag times compared with rumen inoculum (Mauricio et al., 2001, Zicarelli et al., 2011). In pre-weaned calves, increased GP could be indicative of greater microbial activity in faeces, although at week-4 the DMD of starter in faecal incubations was 51 %, compared with 58 % in rumen incubations, possibly suggesting a less efficient, acetogenic, fermentation pathway from the microbes in faeces.

At week-7, rumen inoculum produced a significantly greater volume of gas than faecal inocula, which could indicate increased microbial activity in the rumens of calves about to be weaned. This was not paired with increased DMD in faecal or rumen incubations, which decreased to 47 and 52 %, respectively. In a tandem

study, the *in vivo* total tract digestibility of calf starter was estimated using faecal acid insoluble ash (Van Keulen and Young, 1977). At week-7, DMD was estimated to be 62 %, SEM = 2.31 (data unpublished), demonstrating that rumen inoculum may be more accurate at predicting *in vivo* digestibility. The results of Mauricio et al. (2001) show that the effects of faecal and rumen inocula on *in vitro* feed digestibility in mature cattle are substrate specific, so further investigation into the effect of calf inocula source on the digestibility of other feedstuffs would be valuable. In rumen incubations, modelling estimated significant increases in GP from both rapidly and slowly degradable fractions of calf starter. As with week-4, the rates of degradation of the rapidly and slowly degradable fractions of calf starter, and the lag times of incubations, were similar between inocula, although regression analysis indicated a weak relationship between these parameters. The changes in GP kinetics between weeks four and seven are likely due to the developing gastrointestinal tract and associated microbial dynamics in transition stage calves. If changes in faecal GP could be associated with increasing rumen function in dairy calves, a non-invasive test to inform weaning decisions could be developed. Considerably more data are required to achieve this, however, given the importance of rumen function for a smooth transition from milk to solid feed, it would be a worthwhile area of research.

At week-13, faecal and ruminal inocula produced the least total gas, 36 and 52 ml, respectively, of the three experiments in the study. This was paired with the highest DMD for each inoculum, 55 % for faecal and 60 % for rumen, indicating a potential shift in fermentation to more efficient, less gaseous, pathways. *In vivo* digestibility was estimated to be 68 %, SEM 1.25, showing a similar discrepancy between *in vitro* and *in vivo* digestibility as week seven. As with weeks four and seven, the relationship between faecal and rumen GP kinetic parameters were weak for all parameters. Modelling estimated that faecal inoculum had a significantly longer lag time than rumen inoculum, 2.6 h compared with 1.5 h, and the rate of degradation of the slowly degradable fraction of calf starter was lower in faecal incubations, 0.05 h^{-1} compared with 0.07 h^{-1} in rumen incubations. Six weeks post weaning, these calves should have rumens

functioning in a similar capacity to mature cattle. As such, these results align with studies examining faecal inocula in mature cattle where GP kinetics differ between rumen and faecal inocula (Mauricio et al., 2001).

7.5 Conclusion

These results indicate that there is a weak, or non-existent, relationship between the fermentative activity of microbes in calf faeces and calf rumen digesta. Differences between faecal and rumen inocula GP kinetics, in both pre-weaned and weaned calves, suggests that faeces is unlikely to be a suitable source of microbes to act as a proxy for rumen digesta in calf rumen models. The replication possible in this study was limited by the number of animals and ANKOM^{RF} modules that could be used, and large variations in GP between animals, likely associated with different stages of gastrointestinal tract development, highlights a challenge of refining a model suitable for calves transitioning from pre-ruminant to ruminant. Further examination into the use of faecal inoculum in pre-ruminant, whole tract digestibility models could be worthwhile, but suitable foregut enzymatic digestion steps would likely need defining.

Chapter 8. Summary

The body of research presented in this thesis found limited effects of ANP *in vitro* and no effects *in vivo* in mature cattle. When measuring the effects of five doses of ANP on *in vitro* GP in the first experiment of this thesis, doses of 2 and 5 mg/g DM substrate increased total GP from a grass silage-based ration, potentially indicating an increase in digestibility. When the same ANP doses were incubated with a maize silage-based ration, there was no effect on GP from any dose. This could suggest substrate specificity for ANP efficacy, between grass silage and maize silage, and amylolytic activity, given the grass silage-based TMR was 9.2 % higher in starch. When GP kinetics from grass silage-based incubations were modelled, there were no differences in gas produced from the slowly or rapidly degradable fractions, rates of degradation of each fraction, or lag times. This suggests that the lack of observed effect on GP is not due to substrate-specificity. When VFAs were measured in fermentation fluid from incubations, no differences in fermentation patterns were observed, providing no further insight into the cause of increased GP from the ANP treatments.

Increases in GP do not always mean increases in digestibility. The production of gas *in vitro* is also contributed to by the neutralisation of VFA by the bicarbonate buffer in fermentation media (Mauricio et al., 2001). This means that GP data should always be accompanied by DMD data, as this represents the fermentation of the substrate and the incorporation of that substrate into microbial biomass (Mauricio et al., 2001). In our experiment, significant increases in GP from doses of 2 and 5 mg/g DM ANP were accompanied by numerical increases in DMD. However, as these differences were non-significant, firm conclusions on if ANP had beneficial effects on *in vitro* fermentation, or if it caused a less efficient, more gaseous fermentation, cannot be determined. The analysis of gas composition from ANP incubations would have provided greater insights into its effects on the efficiency of fermentation; with less efficient (and more gaseous) fermentation typically characterised by increases in methane (Wang et al., 2016). As mentioned in Chapter 1, an attempt to collect gas from the ANKOM^{RF}

system in an experiment not included in this thesis was unsuccessful; highlighting an advantage of closed systems such as the Hohenheim gas test to examine the effects of experimental treatments on gas composition (Menke et al., 1979). It is possible to estimate methane production from the VFA profile of *in vitro* fermentation media (France and Dijkstra, 2005, Park et al., 2024). However, as there were no differences in VFAs between incubations in this experiment, such estimates would not have provided greater insights into the effects of ANP on gas composition.

The *in vitro* gas production technique is an advantageous method for assessing the digestibility and digestibility kinetics of feeds, because it is cost effective, rapid, and allows a large number of samples to be tested under highly controlled conditions (Getachew et al., 1998). These advantages also apply to using the technique to investigate the effects of dietary interventions such as ANP. It was selected for use in this research because it enabled the testing of a range of inclusion rates in two dairy rations, with the aim of informing and refining the design of future *in vivo* experimentation. As discussed in Chapter 1, there are several factors that influence *in vitro* GP systems, including the potential and unquantified (in our experiment) effect of donor cow diet on degradation estimates (Rymer et al., 2005). The technique has been used to examine the effects of other *Aspergillus* products on feed digestibility and fermentation kinetics (Facchini et al., 2012, Souza et al., 2021). However, when these interventions have unknown compositions and mechanisms of action, including potentially influencing the rumen microbiome, a limitation of the technique is its inability to mimic the true rumen environment.

Chapters 2 and 5 stress the importance of targeted investigation of fungal fermentation products, where product composition and activities are considered within experimental design. The chemical composition of ANP is proprietary and was not permitted to be investigated in this research, meaning that a targeted approach, such as testing substrates with a complementary nutrient profile for any residual enzymatic actions, was not possible. Alternative methodology to assess ANP *in vitro*, could include catalytic assays (Meale et al., 2014); where residual enzymatic activity could be determined through the measurement of

the rate and quantity of end product accumulation, when ANP is combined with substrates such as cellulose or starch. Additional *in vitro* gas production experimentation, incubating ANP with single substrates, rather than TMR, would provide further insights into if the product has substrate specificity. Pre-treatment of substrates with ANP would also provide insights if it influences feed characteristics prior to rumen fermentation, as similar *A. niger* products have been used successfully as silage inoculants (Xiong et al., 2022).

The data from this preliminary experiment provided little insight into the effects of ANP on rumen fermentation kinetics; it is worth acknowledging that these results may have discouraged some researchers from further investigation into the product. The brief of this project was to investigate the effects of ANP in ruminants, and, as *in vitro* effects (or lack thereof) do not always reflect effects *in vivo*, further experimentation was performed in fistulated cattle.

In vivo, the degradation of grass silage, steam-flaked barley and a dairy TMR formulated to similar specifications as the TMR used *in vitro*, was unaffected by supplementation of ANP at doses of 5, 10 and 15 g/d. Rumen VFA production, rumen pH, apparent total tract digestibility of DM and the degradation kinetics, including effective degradability, of substrate DM, OM, CP and NDF were similarly unaffected. Although the cattle used in this study were a dairy breed (Jersey), their age (12 ± 2 years), weight (659 ± 37 kg) and productivity (non-lactating, non-pregnant), prevent these results from being interpreted in a wider dairy context. Given the physiological condition of these cattle, they were fed a high fibre, straw-based ration, converse to the ration a productive cow would be fed in modern dairy systems (Schingoethe, 2017). The composition of ruminant diets often affects the composition of the rumen microbiome (Newbold and Ramos-Morales, 2020). The rumen microbiome comprises four main taxa: bacteria; protozoa; anaerobic fungi and archaea, and the relative abundance of some microbial species within these taxa changes in adaption to host diet (Belanche et al., 2012). High fibre diets support microbial communities with fibrolytic activities, including bacteria such as *Ruminoccus* and *Fibrobacter* spp. (Belanche et al., 2012), and species of *Piromyces* and *Neocallimastix* fungi (Hartinger and Zebeli, 2021). Whereas high concentrate diets support the

proliferation of microbes with amylolytic and proteolytic activities, such as *Streptococcus bovis* and *Prevotella ruminicola* (Chaucheyras-Durand et al., 2012). The rumen microbiome was not investigated in this project, but, given that cattle were fed diets high in recalcitrant fibre, it is possible that substantial fungal populations were already resident in their rumens (Hartinger and Zebeli, 2021). Filamentous fungi are elite fibre degraders, using hyphae to physically disrupt fibre, and producing bioactive substances that can act as digestibility enhancers (Elghandour et al., 2023). If the cattle used in our study had a sizable rumen fungal population, it is possible that adding small amounts of fungal product (≤ 15 g/d), without a hyphal dimension, would have limited any potential effects from supplementation on rumen fermentation and digestibility kinetics. Notably, the cattle used for the *in vivo* trial were also used as donors for the microbial inoculum used *in vitro*. Straining rumen digesta through layers of cheesecloth for microbial inoculum preparation would likely exclude filamentous fungi from the inoculum. However, endogenous bioactive compounds produced by fungi would have remained, which could have limited the effects of ANP *in vitro*.

The *in sacco* technique enabled the effects of ANP on feed degradability to be examined in the rumen. *In sacco,* the effects of extrinsic factors, such as end product outflow, microbial turnover and changes in pH, provide conditions more representative of those in farmed animals, in comparison to *in vitro* techniques (Vanzant et al., 1998). However, the technique is limited by constraining feeds within bags, meaning they are not exposed to rumination and mastication (Nocek, 1988). Our experimental design followed most of the recommendations of Vanzant et al. (1998); we used a substrate-to-bag surface area of 12.5 mg/cm^2 and bags made of woven, nitrogen free polyester, with a porosity of 50 µm. However, Vanzant et al. (1998) recommends that animals used in *in sacco* experiments are fed a diet similar to that of the feeds being investigated. Due to the physiological condition of the fistulated cattle used in this experiment (overweight), it was not possible to feed them a TMR formulated for high yielding dairy cattle, nor large quantities of barley or grass silage. This is a limitation of the experiment, as the degradation of these feeds and their

interactions with ANP, when suspended in a rumen environment not adapted to degrade them, may have been different from in animals fed a basal diet similar to the incubated substrates.

Aligned with *in vitro* findings, ANP did not affect rumen VFA when measured in fistulated animals. Measuring VFA in the rumen overcomes limitations of the *in vitro* technique, where some VFA are neutralised by buffer and they cannot flow out of the incubation vessel. However, these measurements do not represent absolute VFA production, rather the balance between the rate of production and rate of outflow, which can be influenced by physiology, diet, feeding and microbial ecology (Dijkstra, 1994). France and Dijkstra (2005) suggest that the acetate-to-propionate-to-butyrate ratio in cattle fed high fibre rations should be in the region of 70: 20: 10 and this is similar to the mean ratios observed in our experiment which were 73: 18: 9. If cattle were fed a basal diet of dairy TMR or greater quantities of barley, the fermentation pattern would likely have increased proportions of propionate due to increased levels of dietary starch (France and Dijkstra, 2005). This would have enabled examination of ANP's effect on propionate production in the context of higher energy diets, where, if present, residual amylolytic activities would have had more substrate to act upon.

The *in sacco* technique was selected for use in this research, as it enables both intrinsic and extrinsic factors affecting feed degradation kinetics to be examined. For example, the direct influence of ANP on feeds through catalysing hydrolysis, and any prebiotic effects the product may have on the rumen environment, which could then influence feed fermentation. The technique has been used by other researchers examining the effects of *Aspergillus* products on digestibility kinetics (Caton et al., 1993, Varel and Kreikemeier, 1994, Chiou et al., 2000), indicating its suitability for such investigations. Alternative methodologies such as longitudinal lactation studies, or the use of metabolism chambers, could provide more comprehensive insights into the effects of ANP on dairy cattle productivity and digestive physiology.

In vitro and *in vivo* experiments addressed primary objectives 1 – 3 of this project:

1. Measure the effect of ANP dose on the *in vitro* fermentation kinetics of rations formulated for dairy cattle.
2. Model the effect of ANP on the degradation kinetics of feeds and feed components in the rumen.
3. Assess the impact of ANP inclusion on rumen fermentation pathways *in vitro* and *in vivo*.

Although these objectives have been addressed, they have provided evidence of ANP efficacy, or lack thereof, in a very limited context (geriatric, overweight heifers). An area of future research would be to measure the effect of this ANP in lactating dairy cattle. Some studies have measured improvements in cattle productivity and performance from *Aspergillus* product supplementation (Martins et al., 2022, Cantet et al., 2025). Such research would provide evidence for, or against, ANP use in a more industry applicable context than this research.

As we did not have access to a suitable dairy herd to conduct a lactation study, the remainder of the project was focussed on exploring the use of ANP in dairy calves. In non-ruminants, this ANP has increased digestibility and performance variables (Perić and Spring, 2013, Diógenes et al., 2018). As the digestive system of pre-ruminant calves is similar to that of monogastric animals, we hypothesized that supplemental ANP may be more useful in calves than in mature cattle. Before conducting experimental work, a systematic review of enzymatic product use in calves was conducted to help inform experimental design. This study addressed secondary objective 1:

1. Consolidate and evaluate published literature examining the dietary inclusion of enzymatic products in dairy calves.

This review identified 17 research articles examining the effects of enzymatic products on calf health, performance and welfare. Fifteen of these articles realised a positive effect of experimental treatments on at least one variable, with consistent improvements observed in digestibility studies, suggesting

exploration of ANP in calves was worthwhile. This study addressed primary objective 4:

4. Measure the effects of ANP on growth, feed efficiency, gut health and rumen development in dairy calves.

Most of the studies that I evaluated in the systematic review, that trialled combinations of experimental treatments, did not measure increased effects compared with standalone treatments. However, no previously published data exist on the combination of ANP with MRF. Unpublished commercial trials have measured improvements in calf performance from MRF, as such, the novel combination was trialled in this project, addressing secondary objective 2:

2. Assess any synergistic or additive interactions between ANP and a yeast cell wall fraction when fed to dairy calves in synchrony.

The main finding of this study was that 3 g/d ANP alone, or in combination with 2 g/d MRF, increased the thickness of metabolically active layers in rumen papillae in pre-weaned and weaned periods. This finding would be theoretically consistent with increased absorptive capacity in the rumen (Diao et al., 2019), however, no consistent improvements in FCE or rumen fermentation were observed and no synergistic or additive effects were observed from combining ANP and MRF. Interestingly, ANP calves had increased starter intake over the entire study, and this effect was not observed when ANP was combined with MRF. In the preweaned period, calves fed ANP in combination with MRF had a significantly lower acetate-to-propionate ratio compared to control calves, whereas six weeks post weaning, this shift was observed in calves fed only ANP, paired with a significant increase in the proportion of propionate of tVFA. In the preweaned and transition period, butyrate is widely considered the most important VFA for rumen development, as it is the primary source of energy for epithelial growth and it increases gene expression for papillae maturation (Niwińska et al., 2017). Throughout weaning the molar concentration of butyrate typically increases, altering proportions of acetate-to-propionate-to-butyrate in favour of propionate and butyrate (Niwińska et al., 2017); with Suárez et al. (2007) reporting ratios ranging from 53: 30: 17 to 62: 27: 10 in eight week old

calves. No differences in butyrate were observed in the present study, however, the shift towards propionate was possibly associated with the increases in papillae morphology that we measured. Propionate is the second most important VFA for rumen development in the preweaning period as it provides an energy source for the rumen epithelium, as well as increasing gene expression for papillae proliferation (Diao et al., 2019).

In calves, rumen development and fermentation is driven by microbial colonisation and the intake of solid feeds (Amin et al., 2021). Calves are born with a sterile rumen, which immediately begins to be colonised by bacteria from environmental sources, such as the dam (Ghaffari et al., 2025). Bacteria are the first microbes to inhabit the neonatal rumen, followed by anaerobic fungi and archaea, and finally, protozoa (Malmuthuge et al., 2015). The abundance and diversity of microbes in the preweaned calf is influenced by age, diet and environment (Ghaffari et al., 2025). Li et al. (2012) observed that *Prevotella* bacteria dominate in 14-day old calves, shifting to a *Bacteroides* dominant community by 42 days. The intake of starter feeds rich in starch stimulates the proliferation of amylolytic microbes including *Succinivibrio* and *Streptococcus* spp., whereas the introduction of forage into the calves diet stimulates fibrolytic communities, such as *Ruminococcus* spp (Ghaffari et al., 2025). The preruminant microbiome demonstrates greater plasticity compared to mature ruminants, and early life alterations to the microbiome from some dietary interventions can persist into adulthood (Malmuthuge et al., 2015). This presents an opportunity for strategic use of dietary interventions to programme the rumen microbiome towards more efficient and/or environmentally favourable compositions (Meale et al., 2021).

The rumen microbial composition of calves was not measured in this study, however, MRF supplementation in lambs has been associated with increases in taxa involved in succinate metabolism, suggesting MRF may influence fermentation towards the more efficient propionate pathway (Corrigan et al., 2026). As we observed decreases in the acetate-to-propionate ratio in calves fed MRF in combination with ANP, a valuable area of future research would be to measure if these changes were associated with differences in the rumen

microbiome. The differences in VFA observed in calves, which were not observed in mature cattle or *in vitro,* may also be associated with the increased plasticity of the calf rumen microbiome; where dietary interventions can be more influential on microbial dynamics in early life (Malmuthuge et al., 2015). A limitation of our study design was not offering MRF as a standalone experimental treatment. Although we observed no evidence of antagonism between ANP and MRF, the combination of products makes it impossible to determine if differences observed in rumen variables in the ANP + MRF group were attributable to either, or both, products. A 2 × 2 factorial design would have allowed differences in effects between ANP and MRF to be examined. However, the barn space available for this study was not large enough to house enough calves to maintain sufficient statistical power for an additional experimental treatment group.

The study was further limited by not measuring straw intake, meaning that feed intake measurements were constrained to starter and milk replacer. Given high levels of starter intake, it is reasonable to assume that straw intake was negligible, but, if ANP or MRF altered forage intake and/or digestibility, any effect was not captured due to the experimental design. Another limitation was the age of enrolment of calves. Legally, calves could not be transported to the research farm until they were over two weeks old, meaning that a substantial portion of the pre-ruminant period of our calves occurred without receiving experimental treatments. Products with prebiotic effects can be beneficial in the pre-ruminant period, when calves have higher risk of morbidity (Cangiano et al., 2020). The usual recommendation for products like MRF is to offer them from birth, with Corrigan et al. (2026) observing the best results on ruminant performance when MRF was fed to late gestating animals and subsequent neonates. Notably, there were no cases of morbidity throughout the calf trial, meaning that any potential effects of ANP or MRF during disease, were not observed. This included no effects on calf faecal health, although mean faecal score on the Wisconsin Madison scoring system was low (< 1) for all calves during both preweaned and weaned periods (McGuirk, 2008). Measurement of health and performance variables alone, does not allow for a holistic assessment of calf

welfare. The Five Domains Model states that assessment of the four physical domains (health, nutrition, physical environment, behavioural interactions) is required to assess the affective state of animals (the fifth domain) (Mellor, 2017). By measuring variables in all four domains, animals' emotional state and capacity to have positive experiences, can be evaluated. As such, although calves in our study were healthy and performed well, indicating positive experiences for health and nutrition, we cannot state that they had positive or negative welfare, as we did not measure their behavioural interactions. The systematic review of fungal fermentation product use in calves identified studies in which these products had positive effects on NNOB and resting time (İlhan and Yanar, 2021, Khademi et al., 2022). Behavioural variables can be observed directly or using video or measured using wearable sensors for activities such as lying and standing. The purchase of sensors and video equipment was outwith the budget of our calf trial. Although direct observation would have been possible, it is a potentially less robust method of assessing behaviour; with human presence (Leruste et al., 2013), and the calves' relationship with their stockperson (Ellingsen et al., 2014), influencing their behaviour. Measurement of behavioural variables would have allowed for more comprehensive assessment of ANP and MRF on calf welfare. Given the positive effects we observed on rumen development, it is possible that these products enabled a smoother, and less stressful, transition from liquid to solid feed for calves. The effects of these products on calf welfare would be a valuable area for future research as would examination of the effects of ANP and MRF in calves, when fed from birth, in commercial settings where there is greater potential for disease challenge. Although this author takes great pride in stockmanship, the time spent on cleanliness in this study likely exceeds the time budget available for such tasks on most commercial farms.

The final experiment in this project examined the efficacy of calf faeces as a proxy for rumen digesta to model digestibility in calves. This study used samples from calves in the ANP calf trial, plus five calves that were part of a separate bolt-on study, culled shortly after arrival (aged 27 ± 5 days old). This experiment addressed secondary objective 3:

3. Evaluate if calf faeces could be a suitable proxy for rumen digesta to model calf rumen digestibility in an *in vitro* gas production system.

The use of faecal inocula in gas production systems has been explored in mature cattle and found to be ineffective as a proxy for rumen digesta (Mauricio et al., 2001). However, to our knowledge, the comparison of calf faeces and rumen digesta GP has not previously been investigated. This experiment found sparse similarities between GP kinetics of faecal and rumen digesta incubations at three time-points in the transition stage of calves. Indicating that, aligned with research in mature cattle, faeces is not a useful proxy to model rumen digestibility in calves. A limitation of this study was that there was only capacity for one *in vitro* replication per calf. Given that our calves had an age range of ± 5 days, they would have likely been at different stages of rumen development at each cull. This would have introduced variance between replicas, which, theoretically, could have masked similarities between GP from faeces and rumen inocula from within individual animals. A further limitation was that digesta could only be collected from each animal once, preventing true comparisons over time, as each timepoint modelled a different subset of calves, introducing further variation. This experiment did not produce evidence in favour of using faeces to model rumen digestibility in calves. However, the experimental limitations of this study could be overcome using research that samples the same group of calves over the transition period, using oesophageal tubing, rather than culling, to obtain rumen contents. This would provide more robust evidence for, or against, the use of faeces as a proxy for rumen digesta in calf digestibility models. If this research was repeated, a useful modelling approach would be the method described by Dhanoa et al. (2004), which enables faecal gas profiles to be adjusted in relation to rumen gas profiles. The model then allows degradation in the rumen to be estimated from the kinetic profile of adjusted faecal GP profiles. Using faeces as a proxy for rumen inoculum in the *in vitro* gas production technique does present ethical and financial benefits from removing the need for access to rumen digesta. However, the microbial composition of faeces is fundamentally different as it

contains no protozoa and a different community of archaea (Dhanoa et al., 2004, Mi et al., 2024). This limits faecal GP experiments from estimating ruminal production of methane from feeds and/or dietary interventions, which is an important consideration for any future work.

This PhD project found limited effects of supplemental ANP on *in vitro* digestibility of rations formulated for dairy cattle; no effects of ANP on feed digestibility kinetics or rumen fermentation *in vivo* in mature cattle. I am not aware of research similar to our *in vitro* or *in sacco* studies being performed or published before. However, publication bias towards the reporting of positive results is a significant challenge faced by the academic community. This bias is propagated by a number of factors including increasing pressure upon scientists and journals to publish novel, exciting and high impact research (Song et al., 2013). Indeed, some of the papers included in this thesis were initially rejected by journals for being of limited interest to their audience. However, the publication of null results from robust experiments is important, because these data provide insights into areas of useful research whilst avoiding repetition of research where effects have been limited, and the financial and ethical repercussions of that repetition (especially when the research involves human or animal experimentation). Published research is often the primary evidence base for decision making across disciplines, and the exclusion of null results from this evidence base risks overstating the size, or consistency, of effects, especially when used in meta-analyses. The publication of null results, such as those presented in this thesis, contributes balance to the scientific conversation and allows for comprehensive, evidence-based decisions about product use and future research to be made.

In calves, ANP had a consistent effect on the starter intake and the thickness of metabolically active cells in rumen papillae. Further research of the effects of ANP in calves should be considered a priority and I propose the following questions as worthwhile areas of investigation. Firstly, does ANP affect gut histology beyond the rumen? The physiological integrity of the intestine is important for nutrient absorption and resistance to disease challenge, with neonatal and transition stage calves particularly susceptible to disease (Meale

et al., 2017). Examination of the effects of ANP on mucosal variables such as crypt depth and villi dimensions would provide valuable insights into the use of this product for overall calf gut health and development. Secondly, does ANP influence microbial ecology in the rumen? Examining changes in the microbiome would allow for more comprehensive conclusions to be drawn as to why changes in rumen VFA were observed; either through prebiotic effects or increased starch hydrolysis and propionate production from increased starter intake, or perhaps a combination of both. Finally, does increasing the metabolic capacity of the rumen using ANP improve calf welfare around weaning? Weaning can be a stressful period for calves, especially if their rumens are not sufficiently developed to derive adequate nutrients from solid feed (Drackley, 2008). Quantification of welfare could be achieved using observations of calf NNOB, activity and rumination, and these data paired with performance and health data to provide a holistic welfare assessment. In addition to these research questions, there would be value in measuring the effects of ANP on calf performance (growth, intake, health) under commercial conditions. Although following cohorts of calves on commercial farms does not allow for the same level of control as at research institutes; it does allow for the effects of on-farm environments (such as cleanliness and group housing) to be observed and often enables larger numbers of animals to be enrolled. Although the granularity of data from commercial trials is often limited to high-level performance variables, the familiar context of these trials means that the results are useful to, and sometimes more trusted by, farmers.

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