Growth curves for cattle, and the effect of ammonia-treatment of cereal grains on bovine production performance and faecal proteome

by

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Abstract

The rate of growth of livestock is an important determinant of profitable and environmentally sustainable production systems. Slowly growing animals usually have higher fixed costs of production than rapidly growing animals, and their greenhouse gas emissions intensity (GHG EI) is higher. This thesis investigates the growth of cattle – firstly by investigation of mathematical models of growth, and then by the investigation of the effects of a recently developed method for cereal grain preservation using enzyme-catalysed urea. Finally, the thesis considers faecal proteomic examination as a potential tool to detect health and growth performance differences in cattle. The thesis had two main aims: (1) to find the most suitable growth models for cattle at different life stages using high-density bodyweight data and determine whether the best fitting model(s) improved parameter estimation in comparison with the traditional linear model; (2) to describe the effect of the enzyme-catalysed ammonia treatment of cereal grains on the growth performance of finishing beef cattle and their faecal proteome.

Chapter 2 describes investigations into animal growth models for cattle. Animal growth models can be used to quantify animal growth rates, inform about animal health status, and can serve as the basis for strategies to improve animal productivity and genetic selection. However, model fitting for retrospective performance of animal growth has mostly used relatively infrequent bodyweight (BW) observations. Recent technical developments have enabled multiple records of BW for every single animal every day, automatically weighing animals when they drink milk replacer or water, during milking, or when moving between yards or pens. The logistic, Brody, Gompertz, von Bertalanffy and Richards models were applied to high-density but intermittent bodyweight data from the whole of life of Holstein-Friesian cows; the linear, quadratic, cubic, power and exponential models were fitted to near-continuous BWs of Holstein and Holstein-cross calves for the first 100 days or so of life; the linear, logarithmic and negative exponential models were fitted to near-continuous bodyweights of mixed-breed beef cattle for the last 100 days or so before slaughter. Model comparisons were based on goodness-of-fit statistics and estimations of biological parameters. Generalized linear models were fitted to compare the strength of association between known correlates of growth rate and observed growth rate using two alternative models of growth. Full data sets from the calves and reduced (sparse) data sets were used for comparison of parameter estimate precision derived from the best fitting model and the traditional linear model. The von Bertalanffy model was the best growth

model to describe the whole of life of the lactating Holstein-Friesian cows, the exponential model was the most suitable model for calves, and both the linear and the negative exponential models performed well for fitting to the growth of the finishing beef cattle. Application of the exponential model in calves improved the precision of parameter estimation and the sensitivity of analysis of known correlates of growth compared with the traditional linear model, with both high-density and sparse data.

Chapter 3 describes two trials: one was conducted on a Scottish beef finishing unit where the cattle were fed on a diet based on barley that was treated with ammonia (ATB) or propionate preservatives (PTB), and the other was conducted in a typical Italian beef fattening system where the cattle were fed on a maize-based diet with ammonia treatment (ATM) or without the ammonia treatment (UTM). Consistent with the findings in Chapter 2 that there was little consistent benefit in using non-linear models rather than traditional linear models for analysing growth of cattle in the fattening period, the growth rate estimates in chapter 3 used the traditional method - average daily gain (ADG). Growth performance of animals from groups in each trial were compared, and effects of the treatment on ruminal volatile fatty acids and faeces were studied. The enzyme-catalysed ammonia treatment of grain decreased FCR and faecal starch concentrations, having similar effects of improving the growth performance of cattle in beef fattening systems to those previously reported in studies using direct insufflation with anhydrous ammonia. This method of processing cereal grains has the potential to increase nutrient utilization on commercial cattle farms.

Chapter 4 addresses a potential problem with faecal proteomic studies for cattle: whether highly abundant high molecular weight glycoproteins have detrimental effects on protein identification from faeces that were prepared by filter-aided sample preparation (FASP) method. Therefore, an in-gel sample preparation method (IGSP) was developed, which increased the number of bovine faecal protein identifications.

In Chapter 5, to follow up on the apparent differences in faecal starch and occurrence of diarrhoea in the cattle that were fed ATB or PTB in Chapter 3, the faecal proteomes of a subset of animals were investigated. Faecal samples were analysed by nanoflow ultrahigh-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (nUHPLC-ESI-MS/MS, Orbitrap Elite) after IGSP as developed in Chapter 4, trypsin digestion and TMT labelling. Data were assigned using the Sequest HT search engine to

interrogate sequences in bovine, barley, bacterial (*Clostridium*, *Bacteroides*, *Ruminococcus*, *Prevotella* and *Eubacterium*) and archaeal (30 methanogenic genera) databases in Swissprot and TrEMBL using Proteome Discoverer (PD). Pairwise protein abundance ratios for animals on the two diets were calculated, with the hypothesis test as the background-based *t*-test in PD. Antibodies to bovine serum albumin and barley serpin Z4 were used in western blots (WB) to validate the results from proteomics. In total, 281 bovine proteins, 199 barley proteins, 176 bacterial proteins and 190 archaeal proteins were identified in the bovine faeces. The presence of bovine serum albumin and barley serpin Z4 were confirmed by WB. Mucin 2 was the most abundant host protein identified in the faeces, and many host digestive enzymes and protease inhibitors were also found. Barley serpin Z4 was the most abundant barley protein identified in the faeces. Many microbial proteins were identified in the faecal samples, with a large proportion of bacteria from *Clostridium,* and *Methanobrevibacter* was the dominant archaeal genus. The host proteins were significantly over-represented in biological processes such as microtubule-based movement, defence response to Gram-positive bacterium, negative regulation of endopeptidase activity, cell migration and proteolysis. The microbial proteins were over-represented in biological processes including carbohydrate metabolism, gluconeogenesis, glucose metabolism and glycolysis. Thirty-nine proteins were differentially abundant in the two treatment groups, the majority being more abundant in the PTB group compared to the ATB group (28 *vs* 11).

The investigations described in this thesis identified the best models for growth of cattle at different life stages, and demonstrated the potential to improve the precision of parameter estimations by the most suitable nonlinear models as an alternative to the traditional linear model for calves up to about four months old. The enzyme-catalysed ammonia treatment improved the production performance of finishing beef cattle in two distinct systems. New protocols for faecal proteomic investigation were developed and the effects of enzyme-catalysed ammonia treatment on the faecal proteome were examined. The host, dietary, and microbial proteins of bovine faecal samples in this system were identified, providing a foundation for the future study of cattle GI-related diseases and optimizing diets for cattle to improve performance.

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Author’s Declaration

I confirm that this thesis is my own work, in my own words, and that all sources used in researching it are fully acknowledged and cited. This thesis has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature

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Chapter 1 General Introduction

Accurate estimation of cattle growth is potentially helpful, not only for monitoring animal welfare and on-farm dietary management, but also for research into disease treatments and the development of new zootechnical products. This thesis studied the growth of cattle mainly from two angles: (1) determining the most suitable growth models for cattle at different life stages using high-density bodyweight data, which can provide information on cattle growth characteristics, and can be applied to accurately estimate animal health, predict animal growth and serve as the basis for strategies to improve animal productivity and genetic selection; (2) evaluating the effect of an enzyme-catalysed ammonia treatment of cereal grains on the growth performance of finishing beef cattle and their faecal proteome. The results of the faecal proteomic investigations, used in conjunction with the most precise models for growth should provide a foundation for the identification of cattle growth efficiency markers that might be useful for improving the growth performance. The following content in this chapter will introduce the growth curves of cattle, chemical processing of cereal grains and faecal proteomic studies.

1.1 Growth curves of cattle

Growth trajectories of animals are considered to result from a combination of hereditary and environmental effects and are defined as change in bodyweight (BW) or size over time. The proportions of cattle body tissue change during the growth process, with bone development mainly in the early stage, fat gain in the late stage, and muscle gain in between (Honig et al. 2022). Breed and sex are two main factors that contribute to differences in body composition (Albertí et al. 2008, Venkata Reddy et al. 2015, Honig et al. 2022): beef breeds are characterized by high muscle and low to medium level of fatness while dairy breeds are usually poorly muscled and have a high or medium level of fat; cows and heifers have higher fat levels than steers and bulls. In general, growth curves of animals are sigmoid-shaped, the rate of gain increasing from birth until an inflection point at which the growth rate decreases until the weight reaches a horizontal asymptote or mature weight (Lupi et al. 2016, Rodrigues et al. 2018). Growth curves can be described by nonlinear models, containing biologically meaningful parameters that are moderately heritable, and the analysis of growth curves are therefore useful for the development of genetic selection strategies to suit diverse production systems (Daskiran et al. 2010, Silva et al. 2013, Crispim et al. 2015). From a management perspective, the analysis of growth curves can enhance the assessment of the animal's growth potential, including animals that are in compensatory growth, which is the basis for improving production efficiency (Lupi et al. 2016), and it can enable the early prediction of an animal's ultimate performance (Alonso et al. 2018, Paz et al. 2018, Nascimento et al. 2019). Assessment of the management factors affecting growth, such as feed requirements (Daskiran et al. 2010), detection of physiological disorders of animals preceding the clinical diagnosis of the disease (Maltz et al. 1997), and analysing responses of treatments or response interactions over time (Paz et al. 2018) are all possible through informed analysis of growth curves.

1.1.1 Measurement of bodyweight

In the past, livestock were kept by farmers with small numbers per family, and reliable records of BW were very limited (Widyas et al. 2018). Bodyweight of cattle was most often measured using manual scales set up in races or crushes. There are moving parts in mechanical scales that wear out during use, friction errors caused by dirt within the mechanisms can lead to inaccuracy, and errors are common when reading scales, not only because of movement of animals but also human error. Manually weighing cattle in crushes is not only stressful for the animals, but also labour-intensive for the owners

(Dorota 2010). Digital scales overcome many of the problems of mechanical scales, but precision of weight records remains imperfect. Measurements of BW are also not precise reflections of actual BW because of variation in gut-fill, urine or milk volume. Because BW is the primary measure from which animal growth curves can be derived, there is a need to make it easier to measure BW correctly and at a higher frequency.

Bodyweights of livestock have been estimated from easily accessible morphometric characteristics, such as thoracic circumference, body length, withers height and shoulder width (Coopman et al. 2009, Paz et al. 2018). Researchers developed digital image processing methods to obtain accurate measurements of body condition score (BCS) and estimates of BW (Stajnko et al. 2008, Tasdemir et al. 2011). Dorota (2010) investigated automatic estimation of BW in dairy cows using three-dimensional imaging. Its repeatability, precision and sensitivity were good, and the correlation between BW measured by scale and estimated by camera was high. Maltz et al. (1997) combined individual self-feeders with walk-through scales that measured the BW electronically when the animal was relatively still for a few seconds while eating. Recent technical developments have enabled multiple records of BW to be made on every animal every day, which is becoming increasingly common on commercial farms (Gargiulo et al. 2018, Segerkvist et al. 2020). The automated weighing equipment is often a component of automated feeding or milking systems (Thorup et al. 2012).

1.1.2 Models for animal growth

Animal growth curves can be analysed by different models, depending on the type of animal under consideration and the developmental phase of interest. The logistic (Verhulst 1838), Richards (1959), Gompertz (1825), Brody (1945) and von Bertalanffy (1938) models are the most commonly used models for characterization of growth trajectories, especially over the whole period of growth of animals (Table 1-1). The logistic function was originally used for modelling the growth of human populations rather than of individual organisms, and the Gompertz function was applied to studies on human mortality. Samuel Brody pointed out that the growth curve of animals could be divided into two principal segments, a self-accelerating phase and a self-inhibiting phase, that join during puberty, when the rate of gain is greatest in animals (Brody 1945). The Richards function is an empirical function with one more parameter than the Brody function. However, although it might improve model fitting, the additional parameter increases the

probability of overfitting and of computational difficulties. The von Bertalanffy function was proposed by assuming that the growth rate was the difference between the rate of assimilation and the rate of consumption (Bertalanffy 1938), being considered as the balance of catabolism and anabolism. It was first used for prediction of fish length from age and was then found also to work for weight against age. Now it has been widely used in growth studies of many organisms, including plants. When being used for growth models, these functions use parameters with biological meaning, such as mature BW, integration parameter and maturation rate, which can explain the whole growth process from a biologically significant perspective.

Besides the models mentioned above, the Weibull and Log-normal regression models have also been used to describe growth in male quails (Lucena et al. 2018), and the negative exponential model was applied for modelling growth curves in Moghani sheep (Ghavi Hossein-Zadeh 2017). Other approaches that combined models with machine-learning based on matrix factorization (Alonso et al. 2018), and models with data transformation methods such as quantile regression have also been applied for precise description of growth of animals (Rodrigues et al. 2018, Nascimento et al. 2019).

Table 1-1 The underlying functions for nonlinear models that have been used for livestock growth.

Model	Equation	Instantaneous growth rate	Parameter
Gompertz	$BW = Ae^{-Be^{-kt}}$	$(AkBe^{kt})/(e^{kt}+B)^2$	A: mature BW
Logistic	$BW = A(1+Be^{-kt})^{-1}$	$AkBe^{-Be^{-kt}-kt}$	B: integration parameter
Brody	$BW = A(1-Be^{-kt})$	$AkBe^{-kt}$	k: maturation rate
Richards	$BW = A(1-Be^{-kt})^{m}$	$mAkBe^{-kt}(1-Be^{-kt})^{m-1}$	m: inflection parameter
Von Bertalanffy	$BW = A(1-Be^{-kt})^{3}$	$3AkBe^{-kt}(1-Be^{-kt})^{2}$	

1.1.3 Model fitting and comparison

Nonlinear models as described above can be fitted to summarize the information for the whole of life bodyweight-age data of animals, and the parameters in the models can be

interpreted from a biological point of view. Therefore, these model parameters have been used to estimate breeding values for genetic selection and to intensify the expression of economically important traits. Goodness-of-fit statistics can be applied to determine the best growth model for application under each specific circumstance and in each specific population. The most suitable growth model may not be strictly selected by having the best goodness-of-fit statistics, but it often requires consideration of both goodness-of-fit statistics and the estimates of the biologically significant parameters. In other words, one model might provide excellent goodness-of-fit statistics, while providing biologically nonsensical parameter estimates.

1.1.3.1 Biological parameters in the nonlinear growth models

Most of the parameters in the models represent biological traits of animals or groups of animals. The parameter '*A*' in the models is the asymptotic BW of the animal, and it is interpreted as adult or mature BW. The parameter '*B*' is the integration parameter, which has no direct biological interpretation. Some researchers found it indicated the proportion of the asymptotic BW to be gained after birth (Crispim et al. 2015, Pires et al. 2017), some others suggested it reflected the degree of maturation at birth, the higher value the lower birth weights (Gotuzzo et al. 2019). The parameter '*k*' is generally interpreted as the growth rate of the animal (Gotuzzo et al. 2019). The parameter '*m*' in the Richards model represents shape of the growth curve, that is, the point of inflection at which the phase of deceleration commences just before the adult stature is reached. These parameters are key elements of nonlinear growth models and are indispensable for the best model comparison. A model should be preferred if the predicted values for parameters such as birthweight, mature BW and mature age are closer to the observations.

1.1.3.2 Statistics for evaluation of models

Goodness-of-fit measurement is the key component of model comparison. Statistics such as the coefficient of determination (R^2), adjusted R^2 (R^2_{adj}), mean squared error (MSE), root mean square error (RMSE), mean absolute deviation (MAD), Akaike's information criterion (AIC) and Bayesian information criterion (BIC) have been used commonly to compare the quality of models directly or relatively (Crispim et al. 2015, Pires et al. 2017, Paz et al. 2018). The equations for the derivation of each statistic are as follows (Table 1-2).

Table 1-2. Statistics used for model comparison.

Model	Equation	Interpretation
Coefficient of determination (R^2)	$R^2 = 1 - \sum_{i=1}^{n}(Yi - Yi')^2 \ / \sum_{i=1}^{n}(Yi - \bar{Y})^2$	Yi: observations Yi': predicted values $\bar{Y}$: mean of observations n: number of observations p: number of parameters in model L: maximum likelihood
Adjusted R^2 (R^2_{adj})	$R^2_{adj} = 1 - \ ((n-1) \ / \ (n-p-1)\ )(1 - R^2)$	
Mean squared error (MSE)	$MSE = (1/n) \sum_{i=1}^{n}(Yi - Yi')^2$	
Root MSE (RMSE)	$RMSE = \sqrt{MSE}$	
Akaike information criterion (AIC)	$AIC = \ 2p - 2\ln L$	
Bayesian information criterion (BIC)	$BIC = \ p\ln n \ - \ 2\ln L$	

In statistics, R^2 is the proportion of the variance in the dependent variable which is predictable from the independent variables. It tells how much variance can be explained by the model, providing a measurement of how well the regression predictions approximate the observations. The value of R^2 ranges from 0 to 1, an R^2 of 1 indicating that the predictions perfectly fit the data. However, for a given response, R^2 always increases when adding more explanatory variables, regardless of their true relationship with the response variables. Adjusted R^2 (R^2_{adj}) is less biased towards complex models but does not penalise overfitting sufficiently and strongly enough as a selection criterion, and the value of R^2_{adj} is always ≤ R^2 (Rousson and Goşoniu 2007). R^2 remains a good measurement of model fit when all independent variables in the model affect the dependent variable, or all parameters are significant, and the number of parameters is much lower than the sample size.

Mean square error (MSE) (or mean squared deviation) is the average of squares of residuals - the average squared difference between the estimated values and the observations. A value of MSE close to 0 suggests a good fit of the model. However, Gotuzzo et al. (2019) found it was not a good indicator of goodness-of-fit in their growth models (using Gompertz, logistic and von Bertalanffy growth functions) because of heteroscedasticity (the magnitude of the residuals later in life is much greater than in the initial part). As an indicator of model fit, MSE is relatively more affected by heteroscedasticity than either R^2 or R^2_{adj}. Root mean square error (RMSE) is the standard deviation of the residuals; it tells how concentrated the data is around the predicted curve of the model. Like MSE, RMSE is also sensitive to heteroscedasticity and penalizes larger

errors. It has the same unit as the predictors, and same as MSE: smaller value suggests a greater reliability of the model (Ghavi Hossein-Zadeh 2017, Selvaggi et al. 2017).

Akaike information criterion (AIC) and Bayesian information criterion (BIC) are statistics that are used for comparison of models with different levels of complexity (Aho et al. 2014). For both indices, lower values indicate better models, but they only indicate the relative quality of the models under comparison. They both penalize models with a large number of parameters and the penalty on parameter number is higher in BIC than in AIC (Hojjati and Ghavi Hossein-Zadeh 2018), provided $n \geq 8$. In cases of small sample size, the AIC value might be smaller in a model with more parameters. Thus to address the risk of overfitting, the AICc was developed ($AICc = AIC + (2p^2+2p)/(n-p-1)$), which is essentially AIC with a penalty term for the number of parameters. Researchers found that results from these three statistics (AIC, AICc, BIC) were heavily dependent on the degree of unobserved heterogeneity between data sets and sample sizes (Brewer et al. 2016): BIC performed better if heterogeneity was large while AIC and AICc were likely to perform well when heterogeneity was small; AICc provided a stronger penalty than AIC for smaller sample sizes, and stronger penalty than BIC for very small sample sizes.

1.1.3.3 Growth model comparisons in animal studies

Growth curves of animals are affected by, among other factors, breed, sex (Coutinho et al. 2015, Lupi et al. 2015), population structure (Ghavi Hossein-Zadeh 2015, Hojjati and Ghavi Hossein-Zadeh 2018), management system and environmental conditions, so the best model might be expected to vary with study location, farming system, breed or species. For example, a study of Repartida goats (Pires et al. 2017) showed that the logistic model provided the best average fit (highest R^2, lowest MSE and MAD), the predicted values being more consistent and closer to the actual observations, although it estimated a lower BW at birth. The logistic model also performed the best in fitting the growth of many animals such as Norduz lambs (Daskiran et al. 2010) and Segurena sheep (Lupi et al. 2015, Lupi et al. 2016). However, in a study of Iranian Mehraban sheep (Hojjati and Ghavi Hossein-Zadeh 2018), the logistic model provided the worst fit (lowest values of R_{adj}^2, highest values of DW, RMSE, AIC and BIC), while the Brody model provided the best for this breed. Crispim et al. (2015) proposed that the Brody model would be the best growth model for Brahman cattle, and found it provided more accurate birthweight estimation than the other models. The Richards model was found to be the best growth model for Iranian Shall sheep (Ghavi Hossein-Zadeh 2015) because of the best R_{adj}^2, RMSE, AIC and BIC,

for Podolica bulls (Selvaggi et al. 2017) due to the accuracy in predicting mature BW as well as lower RMSE than the other models, and for Angus cows on pasture because of lowest AIC and BIC, and the best prediction of BWs (Goldberg and Racagnolo 2015). However, this model increases numerical difficulties as it has four parameters, one more parameter than the other growth models. It failed to reach convergence when fitting to the growth of animals such as Morada Nova sheep (Paz et al. 2018) and Brahman cattle (Crispim et al. 2015). For the von Bertalanffy model, it was found to be the best model for Ile de France ewes (Moreira et al. 2016) since it provided the highest R^2 and the lowest error mean square compared to the other models. However, in their study, researchers found that the Brody model estimated the birthweight and BW at the end of trajectory more accurately than the other models. The Gompertz model was proposed to be the most suitable growth model for Raeini Cashmere goats (Ghiasi et al. 2018), however the optimum age at slaughter and mature BW were different from the actual observations. More details of these studies are shown in Table 1-3 below. It should be noted that the greatest frequency of observations was every 15 - 20 days (Lupi et al. 2016).

Table 1-3 Models that have been used to describe the growth curve of farm animals.

Animal	Age	Weighing frequency	Tested models	Model comparison	Best model	Reference
Podolica bulls	0 - 810 d	Every 3 months	Gompertz, logistic, Richards and von Bertalanffy models	Goodness-of-fit (RMSE, R^2, R^2_{adj} and AIC) and BW estimation	Logistic and Richards models (best goodness-of-fit); Richards model (accurate predicted mature BW)	(Selvaggi et al. 2017)
Brahman cattle	0 - 24 m	At birth, 6, 12, 15, 18, 24 months old	Brody, logistic, von Bertalanffy, Gompertz and Richards models	Goodness-of-fit (MSE, R^2, C, MAD and AIC)	Brody model	(Crispim et al. 2015)
Angus cows	0 - 8.1 y	At birth, weaning, 18 months old and every year	Brody, Gompertz, von Bertalanffy, logistic, and Richards models	Goodness-of-fit (-2 log likelihood, AIC and BIC) and BW estimation	Richards model	(Goldberg and Racagnolo 2015)
Segurena sheep	0 - 80 d	Every 15-20 days	Brody, von Bertalanffy, Verhulst, logistic and Gompertz models	Goodness-of-fit (R^2, C, MSE and AIC) and BW estimation	Logistic model (best for biological growth curves); Von Bertalanffy model (best for commercial growth curves)	(Lupi et al. 2015)
Segurena sheep	0 - 80 d	Every 15-20 days	Von Bertalanffy, Verhulst, logistic and Gompertz models	Goodness-of-fit (MD, C and R^2)	Logistic model (best general fit); Verhulst model (best individual fit)	(Lupi et al. 2016)
Ile de France sheep	0 - 210 d	Every month	Brody, von Bertalanffy, logistic and Gompertz models	Goodness-of-fit ($R^2$and MSE)	Von Bertalanffy model	(Moreira et al. 2016)

Repartida goat	0 - 270 d	Every 30 days	Brody, Gompertz, logistic, von Bertalanffy and Richards models	Goodness-of-fit (MSE, R^2, C and MAD)	Logistic model	(Pires et al. 2017)
Mehraban sheep	0 - 365 d	Fewer than 5 records per animal	Brody, Negative exponential, logistic, Gompertz and von Bertalanffy models	Goodness-of-fit (RMSE, R^2_{adj}, DW, AIC and BIC)	Brody model	(Hojjati and Ghavi Hossein-Zadeh 2018)
Shall sheep	0 - 400 d	Fewer than 5 records per animal	Brody, Negative exponential, logistic, Gompertz, von Bertalanffy and Richards models	Goodness-of-fit (RMSE, R^2_{adj}, DW, AIC and BIC)	Richards model	(Ghavi Hossein-Zadeh 2015)
Hemsin sheep	0 - 36 m	At birth, 2, 3, 6, 12, 18, 24 and 36 months old	Negative exponential, Brody, Gompertz, logistic, Bertalanffy, Richards, and Janoschek models	Goodness-of-fit (R^2) and BW estimation	Brody model	(Kopuzlu et al. 2013)
Morada Nova sheep	0 - 730 d	Every 15 days up to 1 year old and every 30 days from 1 to 2 years old	Brody, Richards, von Bertalanffy, Gompertz, and logistic models	Goodness-of-fit (R^2, MSE, MAE and RSD) and BW estimation	Gompertz model	(Paz et al. 2018)
Liangshan pigs	0 - 250 d	20 records per animal	Von Bertalanffy, Gompertz, and logistic models	Goodness-of-fit (R^2)	Von Bertalanffy model	(Luo et al. 2015)

1.1.4 Application of animal growth models

Growth models provide important information about animals, being able to characterize animal performance. A negative relationship between the asymptotic BW (*A*) and maturing rate (*k*) was found in Ile de France female sheep (Moreira et al. 2016), indicating that the animals which had high growth rates tended to have lower asymptotic BW compared to those that had low growth rates. Sex was found to have a significant effect ($p < 0.001$) on these two parameters of Morada Nova sheep (Paz et al. 2018): males always showed higher *A* and *k* parameters than females. Similar findings were reported in Norduz sheep (Daskiran et al. 2010) and Segurena sheep (Lupi et al. 2015) - Norduz male lambs grew faster and attained larger mature BW than female lambs, while the Segurena female sheep had higher growth rate and reached maturity earlier than the males, suggesting value in segregating animals by sex to meet nutritional requirements and determine slaughter age appropriately.

Researchers have proposed the potential of using the improved model parameters to develop optimal selection strategies to achieve desired animal growth patterns (Coutinho et al. 2015, Ghavi Hossein-Zadeh 2017) due to the moderate heritability of the parameters and their correlations with each other. For example, positive genetic correlations between *A* and *k* in pigs have been reported, and researchers identified quantitative trait loci that affected these parameters (Lázaro et al. 2017). Additive genetic variations of biological parameters in the Verhulst and logistic models have been found in Segurena sheep, and were suggested to be introduced as additional selection criteria in breeding programmes (Lupi et al. 2016). Coutinho et al. (2015) selected Nellore cattle by postweaning BWs and found it resulted in the altered growth curves, which also suggested the use of growth models with the biological parameters in selection and genetic improvement programmes. Genetic merit of an outbred pig population has been predicted by analysing the weight-age data, and the growth curves were constructed which incorporated genomic estimated breeding values (gEBVs) and identified the most relevant single nucleotide polymorphisms (SNPs) associated with and likely candidate genes influencing growth model parameters (Silva et al. 2013, Silva et al. 2017). Crispim et al. (2015) identified SNPs associated with phenotypes based on growth model parameters in a multi-trait genome-wide association study (GWAS) of Brahman cattle, which informed the search for causative mutations influencing growth rates. Therefore, using estimated parameters from more accurate growth models as selection criteria would be expected to improve the rate of selection.

More accurate growth models enable more precise management compared with that achieved using inaccurate models. Based on the best growth model and the relative expression level of growth-related genes, researchers determined the most suitable slaughter-weight for Liangshan pigs (Luo et al. 2015). Analysing the biological parameters of the best model representing growth curves of Repartida goats (Pires et al. 2017), researchers found that the animals had adapted to the adverse condition of environment and suggested that nutritional strategies should be implemented after weaning, and animals could be slaughtered before 210 days of age due to their slow growth rate at maturity. The potential to alter growth curves of Irish beef cattle to meet specific breeding objectives had been proposed. Early warning of anomalies was also possible and guidelines could be provided to farmers based on the most suitable growth models of animals (Widyas et al. 2018).

1.2 Chemical processing of cereal grains

In Europe, cereal grains contribute a large proportion of the diets for fattening cattle, and particularly in northern and western Europe, they are often harvested with a high moisture content, necessitating some form of preservation to prevent spoilage by microbial growth (Olsson et al. 2002). Many physical approaches have been taken, such as controlling moisture and temperature, and creating an anaerobic environment which is not conducive to survival and reproduction of microorganisms and pests (Hashem et al. 2012, Navarro 2012). In order to not only inhibit contamination but also improve nutritional value and promote nutrient utilization, some chemical methods have been applied to cereal grains alone or in combination with physical methods (Muck et al. 2018). However, excessive use or processing of grains can be harmful to animal health, for example by accelerating ruminal starch degradation and increasing risk of rumen fermentation disorders (Humer and Zebeli 2017). Finding an ideal grain processing method that can have as many of the previously mentioned benefits as possible without compromising animal health has been an important research topic.

1.2.1 Acid treatment of grains

Many acids, including formic acid, sorbic acid and benzoic acid, have been used for grain storage (Raeker 1990). Organic acids such as propionic acid, lactic acid and acetic acid are naturally present in the gastrointestinal (GI) tract, so have been recommended on the grounds that they are expected to be safe for users and animals (Castillo et al. 2004). In addition to inhibiting microbial growth, acidification can improve protein preservation, silage aerobic stability and nutrient characteristics of grains, thereby improving animal performance (Humer and Zebeli 2017, Muck et al. 2018).

Gheller et al. (2020) found that acid treatments can maintain the temperature of grains for a long period, avoiding the temperature increase which is associated with the growth of undesirable microorganisms. They also directly inhibit contamination. For example, formic acid, sorbic acid and benzoic acid can directly suppress spoilage bacteria and inhibit moulds and yeasts (Muck et al. 2018). Nadeau (2007) reported a lower level of production of lactic acid and ethanol in grains treated with formic acid or propionic acid, resulting in better preservation. Propionic acid has been widely used to preserve and process high-moisture grain for decades (Horton and Holmes 1975). Like the other acids, it can inhibit

contamination (Jones et al. 1970, Goering and Gordon 1973, Raeker 1990) and improve aerobic stability (Wang et al. 2017). For example, researchers found that a buffered propionic acid-based additive prevented yeasts and production of butyric acid in barley and maize, improving their aerobic stability (Kung and Ranjit 2001, Kung et al. 2004).

The effects of acid treatment of cereal grain on animal performance have also been studied. Jones et al. (1970) found that average daily gain (ADG) and milk protein production of dairy cows that were fed on propionic acid-treated maize were improved, and milk fat percentage was decreased; for the heifers and the pigs, there was increased feed efficiency (FE) with no observable side effects on animal health. Similarly, Horton and Holmes (1975) reported increased ADG in beef cattle that were fed rolled propionic acid-treated maize. Gheller et al. (2020) found an increase in feed intake (FI) of dairy cows, as well as improvements in fat-corrected milk production and milk protein when a propionic acid-treated total mixed ration (TMR) was fed.

1.2.2 Alkali treatment of grains

1.2.2.1 Effects of alkali treatment on grains

Many processing approaches to animal feed are based on alkalis such as sodium hydroxide (NaOH), urea and ammonia (Campling 1991). The alkali treatments can prevent mould growth on moist grain (Bothast et al. 1972, Ørskov 1979, Deschard et al. 1987, Kabak et al. 2006) and increase their pH (Anderson et al. 1981, Deschard et al. 1987). Compared to untreated grains, treatment of NaOH was reported to increase the pH of high-moisture maize (11.94 *vs* 4.53) (Anderson et al. 1981) and barley (10.2 *vs* 5.4) (Kennedy and Rice 1987), so did ammonium hydroxide (NH_4OH) in maize (8.38) (Anderson et al. 1981). High pH values of alkali-treated whole-crop wheat silages of 9.27, 8.93, 8.91 and 8.66 following treatment with NaOH, NaOH combined with urea, urea and ammonia, respectively, compared to the pH of 4.88 in untreated wheat (Deschard et al. 1987). Like acid treatment, alkali treatment was also expected to improve nutrient characteristics of grains. Increased dry matter (DM), gross energy, neutral detergent fibre (NDF) and acid detergent fibre (ADF) were found in the whole-crop wheat silages that were treated with NaOH, urea, or combination of both (Deschard et al. 1987). However, adverse effects of NaOH treatment such as reduction in vitamin E, lysine and cysteine have been reported (McNiven et al. 1995, Dehghan-Banadaky et al. 2008). In addition to protecting grains

from contamination (Bothast et al. 1972, Kabak et al. 2006), treatment with ammonia enhances the nutrient value as well. The addition of ammonia has been widely used to increase non-protein nitrogen (NPN) (Horton 1978, Herrera-Saldana et al. 1982, Males and Gaskins 1982, Kraiem et al. 1991), which contributes to improved reticuloruminal microbial growth and activity (Rode et al. 1986), and might therefore improve animal performance (Spanghero et al. 2017, Belanche et al. 2021). A commercially available method (Harbro limited, Turriff, Scotland) of cereal grain preservation using enzyme to catalyse the conversion of urea to ammonia has also been developed. Not like the previous method that using anhydrous ammonia gas, grain is mixed with urea and a source of urease, together with sufficient water to allow the reaction to occur, then deposited in commodity bays and covered with plastic sheeting for 7-10 days, during which ammonia gas percolates through the cereal grains and is absorbed by the grain.

1.2.2.2 Effects of alkali treatment on digestibility and rumen environment

Alkali treatment was originally used to increase the digestibility of low quality roughages (Jackson 1977, Oji et al. 1977, Han et al. 1978, Herrera-Saldana et al. 1982, Kraiem et al. 1991), and has also been used for cereal grains (Laksesvela 1981). Sodium hydroxide hydrolyses hemicelluloses and lignin, thus improving fibre digestibility (Berger et al. 1981, McNiven et al. 1995). Ammonium hydroxide can improve the digestibility as well, but the improvements of NaOH in digestibility of grains were reported to be greater than those from NH_4OH (Berger et al. 1981). Researchers found that the increased digestibility of cereal grains from alkali treatment was similar to that achieved by rolling or crushing (Ørskov and Greenhalgh 1977), and recommended the use of alkali treatment instead of mechanical treatment to save costs (Humer and Zebeli 2017). However, other researchers found that there were no effects of NaOH, NaOH combined with urea, urea or anhydrous ammonia treatments of wheat silage on apparent digestibility of organic matter (OM), and the decreased digestibility of starch tended to offset the increase in fibre digestibility (Deschard et al. 1987). McNiven et al. (1995) reported that the treatment of NaOH of barley lower the starch digestibility in the rumen and small intestine of lactating cows compared to those fed rolled or roasted barley. Increased DM digestibility of high-moisture maize and ruminal fibre degradation resulted from the NaOH treatment have been reported by Anderson et al. (1981), while no changes resulted from the NH_4OH treatment compared to the untreated diet were found. Rode et al. (1986) reported that there was no significant effect of urea or anhydrous ammonia treatment on *in vivo* DM digestibility of high-

moisture barley, but higher apparent digestibility of starch and ADF were found compared to the untreated diet. In their study, *in sacco* DM degradation was greater after 8 hours' incubation in the rumen when the diet included urea- and ammonia-treated compared to untreated barley.

Alkali treatment can increase ruminal pH values (Anderson et al. 1981) and reduce ruminal pH fluctuations (McNiven et al. 1995). The ruminal pH of cows that were fed with NaOH-treated maize was increased compared to those fed the untreated diet (6.34 *vs* 5.83) (Anderson et al. 1981). Higher ruminal pH was noted in sheep that were fed on ammonia-treated grains compared to those fed on untreated barley (7.1 *vs* 6.6 after feeding for 4 weeks) (Laksesvela 1981) or barley supplemented with urea (6.24 *vs* 5.99) (Belanche et al. 2021). However, Robinson and Kennelly (1988) found that the ruminal pH of dairy cows declined slightly (still above 6.09) as the level of ammoniation increased in treating barley. In their studies (Robinson and Kennelly 1988, 1989), the ammonia treatment increased ruminal acetate, butyrate and degradable NDF, but the rumen NDF in OM declined as the level of ammonia treatment increased, and no effects of the ammonia treatment on degradable DM and digestibility of OM, NDF or starch were found. Mandell et al. (1988) reported increased degradation of ruminal crude protein (CP) and decreased degradation of ruminal OM in steers that were fed ammonia-treated barley. Ruminal propionate was increased and butyrate was decreased, while there were no effects on ruminal NDF degradation, total VFA or acetate. Another important effect of the alkali treatment on animals is to slow the degradation rate of starch in the rumen, thus lowering the risk of rumen fermentation disorders (Humer and Zebeli 2017). A low rate of enzymatic glucose release (Srivastava and Mowat 1980) and ammonia production in rumen (Nikulina et al. 2018) after ammonia treatment of cereals were reported, which might provide more balanced fermentation and more stable conditions for microbial protein synthesis, improving nutrient utilization (Ørskov and Greenhalgh 1977, Ørskov 1979) as well as lowering the risk of rumen acidosis.

1.2.2.3 Effects of alkali treatment on animal performance

Higher liveweight gain and feed intake in steers that were fed NaOH-treated wheat silage compared to those fed untreated diet was found (Deschard et al. 1987). However, there were contrasting findings of the effects of NaOH treatment on animal intake (Ørskov et al. 1978, McNiven et al. 1995). Average daily gain and FE were reduced in steers that were

fed NaOH-treated maize (Anderson et al. 1981). There were increases in water intake and urination due to alkali treatment of grains, which resulted in an increased requirement for straw bedding (Ørskov 1979, Deschard et al. 1987). Although Barnes and Ørskov (1981) did not find problems with animal health (livers and kidneys of slaughtered animals were normal after animals were fed on 45 g/kg NaOH-treated barley over more than ten days), the potential adverse effects on animals such as nephrotoxicity after prolonged feeding of 87.5 % NaOH-treated barley, possibly resulted from high sodium content and pH value and the presence of lysinoalanine (Kennedy and Rice 1987), and risks to farmers as well as possible caustic burns in animals still require attention.

With ammonia treatment, the possibility of palatability problems should also be considered (Mandell et al. 1988), but can be solved by exposure to the air for some days prior to feeding (Rode et al. 1986). The effects on animal performance have been inconsistent among studies (Table 1-4). In sheep, increases in DMI and ADG, with improved reproductive capacity, were reported if they were fed ammonia-treated barley compared to untreated barley (Laksesvela 1981). Researchers found increases of ADG and FE in steers (Mathison et al. 1989), improvement of milk yield and production of milk protein and lactose in dairy cows that were fed ammonia-treated barley (Robinson and Kennelly 1989) and increases in growth rate, DMI and final weights of steers that were fed ammonia-treated maize (Phillip et al. 1985). Mathison et al. (1989) also reported an increase in carcass weight of steers that were fed on ammonia-treated barley compared to those fed on untreated barley. However, no differences in FE of steers in the growing phase, or ADG and FE in the finishing phase were found between animals that were fed ammonia-treated and untreated barley (Bradshaw et al. 1996). Likewise, ammonia treatment of barley had no effect on DMI, ADG, FE and carcass traits in steers (Yaremcio et al. 1991), nor on DMI of steers that fed ammonia-treated maize (Mowat et al. 1981).

Table 1-4. The effects of ammonia treatment on grains and animals.

Grain	Species	Processing method	Comparison	Effects on grain	Effects on animal	Reference
Barley	Sheep	Adding enzyme-catalysed urea (Maxammon)	Maxammon-treated barley *versus* barley supplemented with urea immediately pre-feeding	Increase: total N content	Decrease: effective rumen degradable N, rumen pH and acetate molar proportion, total apparent N digestibility and urinary excretion of purine derivatives	(Belanche et al. 2021)
Barley	Steers	Adding anhydrous ammonia Rolling	Ammoniated whole barley *versus* non-ammoniated whole barley Ammoniated rolled barley *versus* non-ammoniated rolled barley	No effect: nutrient composition	Increase: (trend) ADG and FE No effect: carcass traits and grades	(Goonewardene et al., 1998)
Barley	Steers	Adding anhydrous ammonia Tempering and rolling	Tempered ammoniated rolled barley *versus* tempered rolled barley supplemented with urea	-	Decrease: apparent digestibility of dry matter and gross energy No effect: ADG, FE, longissimus muscle area and kidney-pelvic-hear fat	(Bradshaw et al. 1996)

Barley	Dairy cows	Adding anhydrous ammonia	Different levels of ammonia-treated barley *versus* non-ammoniated barley	-	Increase: N digestion (trend), milk yield and production of milk protein and lactose Decrease: proportion of NDF in rumen OM No effect: feed intake, rumen bacterial composition, apparent digestibility of OM, NDF and starch, total rumen pool sizes of wet ingesta and DM	(Robinson and Kennelly 1989)
Barley	Steers	Adding anhydrous ammonia	Different levels of ammonia-treated barley *versus* non-ammoniated barley	Increase: CP, ammonia N, ADF, acid detergent insoluble nitrogen and acetic acid Decrease: lactic acid, moulds and yeasts No effect: aerobic bacteria	Increase: final weight, carcass weight, DMI, ADG and FE Decrease: digestibility of DM, OM and energy	(Mathison et al. 1989)
Barley	Dairy cows	Adding anhydrous ammonia	Different levels of ammonia-treated barley *versus* non-ammoniated barley	Increase: N content Decrease: NDF content	Increase: whole-tract true DM digestion and size of degradable fraction Decrease: rate of degradable DM fraction degradation No effect: rate of ruminal N release and N digestion	(Robinson and Kennelly 1988)

Barley	Dairy cows	Adding anhydrous ammonia	Different levels of ammonia-treated barley *versus* non-ammoniated barley	Increase: total and ammonia N content and lignin	Increase: feeding speed, neutral detergent residue degradation and rumen acetate and butyrate Decrease: rumen pH, rate of degradable fraction degradation, 3 methyl butyrate content and size of undegradable fraction No effect: soluble and degradable DM fraction sizes, DMI	(Robinson and Kennelly 1988)
Maize	Steers	Adding anhydrous ammonia	Ammoniated maize *versus* non-ammoniated maize	Increase: protein N, aerobic stability and pH Decrease: total free-amino-acid-N and DM loss	Increase: final weight, growth rate, DMI and OMI	(Phillip et al. 1985)
Barley	Sheep	Adding anhydrous ammonia	Ammoniated barley *versus* non-ammoniated barley	-	Increase: digestibility of DM, OM and crude fibre, weight gain, pH of ruminal fluid and reproductive capacity	(Laksesvela 1981)

The effects shown in the table were derived from experimental results when using only ammonia treatment as the independent variable. Maxammon (Harbro Ltd.): combined grains with urea and enzymes that catalysed the conversion of urea to ammonia.

1.3 Faecal proteomics studies

Faeces accumulates proteins, peptides, lipids, and carbohydrates due to leakage, exfoliation and secretion, as it constantly samples the environment it is exposed to when passing down the gastrointestinal tract (GIT) (Ang et al. 2017, Palomba et al. 2018, Nice 2020). Theoretically, most proteins in the faeces can be detected if proper methods are used to extract and detect proteins and the databases are suitable. Characterization of faecal proteins has the potential to reveal host responses to changes such as digestion and diseases, and has been applied to study of dietary composition (Sistiaga et al. 2014), biomarkers of diseases (Ang et al. 2017) and precision medicine (Jin et al. 2017). Metabolome is also useful but potentially less useful as a biomarker. For example, proteins lend themselves to diagnosis via lateral flow tests because immunoassays are relatively simple. For farm animals, information from faeces might also indicate the efficiency of management and ration formulation. Its non-invasive and easy collection method makes faeces a popular research subject, and repeated sampling over a short period of time is possible. For GI-related diseases, faeces has been recommended as an alternative to blood for diagnosis (Ang et al., 2017), since some specific components in the faeces that come from GI tumours or other epithelial lesions might be at relatively higher concentrations than those in blood or urine due to the close proximity to the lesion and lack of dilution by uptake and distribution in plasma (Nice 2020).

1.3.1 Technologies for studying proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), is the most important technology for analytical protein separation. Whereas one-dimensional gel electrophoresis (1-DE) separates proteins according to their molecular mass, two-dimensional gel electrophoresis (2-DE) can separate proteins according to both net charge and molecular mass (Graves and Haystead 2002). The 2-DE technology allows proteins to be identified and quantitatively compared in samples, with the presence and absence of spots indicating qualitative protein expression, and the intensity of spots showing quantitative information. However, only one sample can be analysed per gel and the number and the type of proteins that can be resolved by 2-DE are limited; the detection of low-abundance proteins can be very difficult when highly abundant proteins dominate the gel. Western blot (WB) and enzyme-linked immunosorbent assay (ELISA) are mature techniques in molecular biology that rely on specific antibody to proteins of interest and

have been widely used to detect and quantify specific proteins. Both techniques are commonly used to validate the results from proteomic studies.

The term “proteomics” was first proposed by Marc Wilkins et al. in 1990s. The most common proteomics experiment is ‘bottom-up proteomics’, in which proteins are identified by sequencing the digested peptide fragments, which can also be used to determine the protein modifications (Lippolis et al. 2019). In addition to sample preparation, which will be discussed later, there are two main steps in proteomics: sample ionization and mass analysis. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two main methods used to ionize peptides with addition or loss of one or more protons, and then deliver the sample to the mass spectrometer (MS) for further analysis (Graves and Haystead 2002). Electrospray sources have been used to connect with liquid chromatography (LC) that automatically purify and deliver samples to MS (Graves and Haystead 2002), while in-gel digested proteins ionized by MALDI can be used directly in MS without chromatographic separation (Qin et al. 1997). Liquid chromatography and gas chromatography (GC) are the two types of chromatography techniques, but unlike LC, GC is limited to volatile samples. Time-of-flight, ion trap, orbitrap, quadrupole and Fourier transform ion cyclotron are commonly used mass analysers (Aebersold and Mann 2003). Two or more mass analysers can be coupled together (tandem mass spectrometry, MS/MS) to increase their abilities in analysis, which has been applied in many studies to detect sample proteins based on the identified peptide sequences. There are approaches that allow protein quantification in addition to identification in MS-based proteomics, including isotope-coded affinity tags (ICAT), isobaric labelling (iTRAQ and TMT) and stable isotope labelling with amino acids in cell culture (SILAC).

1.3.2 Sample preparation for faecal proteomics

Researchers found small differences in microbiota between voided faecal samples and rectal swab samples (Bassis et al. 2017). Identification of faecal components can be significantly affected by differences in sample preparation approaches (Tanca et al. 2015) and storage matrix (Morris and Marchesi 2016). Morris and Marchesi (2016) suggested that the faecal proteins should be stored in an intact frozen faecal matrix to provide more stable protein levels and protease activity, compared with extracted protein in solution. The buffers used to extract faecal protein varied from study to study. For example, phosphate

buffered saline (PBS) (Cerquetella et al. 2019, Cerquetella et al. 2021), sodium dodecyl sulfate (SDS) (Tanca et al. 2014, Tanca et al. 2017, Liu et al. 2018) and Tris (50 mM Tris-HCl with 10mM $CaCl_2$, pH 7.8) (Debyser et al. 2016, O' Reilly et al. 2021) have been used in studies. No significant effect of addition of 0.05% (w/v) NaN_3 in PBS-based buffer on protease activity was found, and thus it was proposed for long-term extracted faecal protein storage (Morris and Marchesi 2016). Zhang et al. (2020) recommended using SDS-based lysis buffer in combination with ultrasonication in gut metaproteomic studies since the method achieved higher protein extraction yields and protein identifications than using the commercial bacterial protein extraction reagent or the urea-based lysis buffer. In addition, they found that bead-beating increased protein extraction yields compared to extraction without bead-beating, in line with the finding of Morris and Marches (2016). Other mechanical cell disruption methods such as heating and freeze-thawing have also been applied in metaproteomic studies to facilitate protein extractions (Tanca et al. 2014). Differential centrifugation was also found to increase microbial protein identification, reduce host- and food-derived proteins, which affected functional and structural information of taxonomies (Tanca et al. 2015).

Filter-aided sample preparation (FASP) is a common method for the generation of tryptic peptides prior to LC-MS/MS (Wisniewski et al. 2009). It has also been used in faecal proteomics studies of human (Zhang et al. 2018), dog (O' Reilly et al. 2021) and sheep (Tanca et al. 2017, Palomba et al. 2018). Faeces is a complex, heterogeneous, mixture of compounds with a huge range of small, potentially chemically active molecules. A clean-up process prior to protein digestion or LC-MS/MS might be needed to remove substances which affect the downstream analysis. One-dimensional SDS-PAGE could fractionate complex sample proteomes and clean up samples with little loss, which could also be used to select proteins of interest prior to protease digestion (Goldman et al. 2019). In-gel protein digestion has been used in faecal proteomics studies of humans (Ang and Nice 2010, Debyser et al. 2016, Bosch et al. 2017) and mice (Oleksiewicz et al. 2005, Ang et al. 2010). Some researchers used a commercial kit to clean up the extracted faecal protein before 2-DE, and digested the differentially expressed protein spots for LC-MS/MS (Cerquetella et al. 2019, Cerquetella et al. 2021).

1.3.3 Host proteins in faeces

1.3.3.1 Host proteins in human faeces

Faecal proteomics is relatively more studied in human medicine than in other species. With the current level of instrument sensitivity, more than 600 human proteins can be detected in faeces (Bosch et al. 2017, Nice 2020). Most of the human faecal proteomics studies to date have been focused on disease biomarkers and classification. Calprotectin (S100 family) is stable in faeces and has been proposed as a biomarker for human inflammatory bowel disease because its concentration in faeces reflects the intensity of the neutrophilic infiltrate in the gut mucosa (Roseth et al. 1996, Lehmann et al. 2015). Patients with steroid-refractory GI acute graft versus host disease had higher faecal calprotectin levels than patients with steroid-responsive disease (Broglie et al. 2018). Colorectal cancer (CRC) is one of the diseases that has been most studied using faecal proteomic techniques. Ang and Nice (2010) found that haemoglobin, myeloperoxidase, S100A9, filamin A and L-plastin were present at high levels only in the CRC patients. Moreover, compared to the healthy volunteers, α-1-antitrypsin, α-1-acid glycoprotein, C3, fibrinogen, haptoglobin, haemoglobin α and β subunits (HBA and HBB), myeloblastin and transferrin were only found in faeces of the CRC patients (Ang et al. 2011). Glucose-6-phosphate isomerase, lactate dehydrogenase A, transketolase, and transaldolase 1, which are frequently observed in neoplastic cells involved in the reprogramming of cancer cell metabolism and aerobic glycolysis, were expressed significantly differently in faeces of CRC patients relative to healthy controls (Bosch et al. 2017). Eight members of the complement system, which has been reported to function in both immune response and immunosuppression to cancer, were also identified in faeces of CRC patients, with three complement members (C3, C5 and C9) among the top 29 candidate biomarkers of CRC (Bosch et al. 2017). In addition to the already mentioned proteins, Bosch et al. (2017) proposed that proteins including lactotransferrin, hemopexin, myeloperoxidase (MPO), serpin family F member 2 (SERPINF2), cytidine deaminase, azurocidin 1 (AZU1), retinol binding protein 4, fibronectin 1 and glutathione-disulfide reductase (GSR) might also be biomarkers of CRC. In their study (Bosch et al. 2017), they also found that concentrations of C3, S100A8/A9, HBB, SERPINF2, transferrin and GSR in the faeces of patients with advanced neoplasia were higher than those in the control samples; and SERPINF2, LTF, hemopexin, MPO and AZU1 were found to be differential between patients with advanced adenoma and control samples. Debyser et al. (2016) found that the most abundant human proteins in the faecal

proteomes of cystic fibrosis patients were acute phase proteins associated with inflammation. In their study, human proteins such as carbonic anhydrase 1, merprin A β subunit, triosephosphate isomerase, angiotensin-converting enzyme 2, orosomucoid 1, zinc-α-2-glycoprotein, spasmolytic protein trefoil factor 2, cytochrome c and neprilysin were found only in faeces of the patients, while proteins such as pancreatic zymogen granule membrane protein GP-2, elastase 3A pancreatic, phospholipase A2, chymotrypsin-C were only found in faeces of the patients' healthy siblings.

1.3.3.2 Faecal proteins of other species

Faecal proteins of mouse have also received some attention in GI research because of its intensive study as an animal model of human diseases. By using 2-DE combined with MALDI-MS/MS, researchers (Oleksiewicz et al. 2005) identified serum albumin, IgG Fc binding protein, pancreatic amylase 2, secreted carbonic anhydrase VI, pancreatic elastase 3B, pancreatic carboxypeptidase B1, α-2μ-globulin, mucin and trypsin in healthy mouse faeces. A total of 336 proteins in murine faeces were identified by using LC-MS/MS (Ang et al. 2010), among which there were 115 murine proteins, including CRC-associated proteins such as haemoglobin, haptoglobin, hemopexin, α-2-macroglobulin and cadherin-17; the other proteins were bacterial, dietary and parasitic proteins. There have been few faecal proteomics studies of other species. Compared to healthy dogs, Cerquetella et al. (2019) found that immunoglobulin J-chain isoform 1 only presented in dogs with food responsive diarrhoea (FRD), which might be explained by increased activation of the immune system or mucosal damage, or both, in diseased dogs. They (Cerquetella et al. 2021) also identified some faecal proteins from healthy cats and dogs by using 2-DE and LC-MS/MS. Our previous study (O' Reilly et al. 2021) identified and characterized the potential biomarkers that could differentiate among canine chronic enteropathies. There were 98 proteins in faeces of dogs with diarrhoea that were significantly differently abundant than in healthy dogs. Immunoglobulins were more abundant in healthy dogs, whereas acute phase proteins were higher in dogs with diarrhoea. Significantly higher abundances of haemoglobin and fibrinogen were noted in dogs with antibiotic responsive diarrhoea (ARD). Proteins such as haptoglobin, S100A8/9, lactoferrin, α-1 antitrypsin and lysozyme were found to be more abundant in dogs with ARD compared to the dogs with inflammatory bowel disease (IBD). Palomba et al. (2018) identified 431 proteins in sheep faeces, including complement system members and regulators, members of the S100 family, peptidoglycan recognition protein and Ig alpha-1 chain C region (part of IgA),

which are involved in immune and inflammatory response. They found that over 15% of the faecal proteins were peptidases (such as azurocidin and metallopeptidase families), which are involved in many different biological processes, including digestion, immune response and apoptosis. They also concluded that the most abundant protein family in sheep and human faeces was the intermediate filament family. Liu et al. (2018) identified 21 proteins in tick faeces, 18 of which were tick origin, including actin, enolase, AV422, histone H2B, serpin and paramyosin, while the other three proteins, namely serum albumin, HBA and HBB, were likely from the host (hedgehogs). The identified tick proteins might play a role in hindering blood clotting, immune mediation and resistance to bacteria, as well as the formation of muscle tissue in ticks. Studies about identification of faecal host proteins are listed in Table 1-5.

Table 1-5. Faecal proteomics studies.

Species	Objective	Approach	Reference
Dog	Find biomarkers that might aid diagnosis of chronic diarrhoea and distinguish aetiologies among ARD, FRD and IBD.	TMT-based LC-MS/MS	(O' Reilly et al. 2021)
Dog and cat	Identify proteins present in healthy dogs and cats.	2-DE and LC-MS/MS of in-gel digested proteins	(Cerquetella et al. 2021)
Dog	Identify abundant proteins in dog faeces and to find potential diagnostic markers of FRD.	Quantitative analysis of 2-DE and LC-MS/MS of in-gel digested proteins	(Cerquetella et al. 2019)
Monkey	Develop a framework of faecal proteomics to study the behaviour and physiology of the host.	Label-free LC-MS/MS	(Tsutaya et al. 2021)
Sheep	Explore proteins secreted in the sheep intestinal lumen.	Label-free quantification LC-MS/MS	(Palomba et al. 2018)
Tick	Explore the faecal proteomes of the tick Haemaphysalis flava.	Label-free LC-MS/MS	(Liu et al. 2018)
Human	Find if there are proteins in the faeces that outperform or complement haemoglobin in detecting CRC and advanced adenomas.	1-DE and label-free quantification LC-MS/MS of in-gel digested proteins	(Bosch et al. 2017)
Human	Explore the host and microbial protein composition of the GIT and their functional changes resulting from CF.	1-DE and label-free LC-MS/MS of in-gel digested proteins	(Debyser et al. 2016)
Human	Show the potential of a hypothesis-driven approach for rapid and quantitative CRC biomarker discovery.	1-DE and LC-MS/MS with MRM mode of in-gel targeted proteins	(Ang and Nice 2010)
Mice	Detect potential biomarkers of CRC based on mice models.	1-DE and label-free LC-MS/MS of in-gel digested proteins	(Ang et al. 2010)
Mice	Establish a diagnostic method to detect GI disease-associated faecal proteins.	2-DE and label-free MALDI-MS/MS of in-gel digested proteins	(Oleksiewicz et al. 2005)

Approaches that were not indicated using in-gel digested proteins were based on filter-aided sample preparation; LC-MS/MS: Liquid chromatography with tandem mass spectrometry; FRD: Food responsive diarrhoea; 2-DE: Two-dimensional polyacrylamide gel electrophoresis; ARD: Antibiotic responsive diarrhoea; IBD: Inflammatory bowel disease; TMT: Tandem mass tag; CRC: Colorectal cancer; 1-DE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (one-dimensional SDS-PAGE); GIT: gastrointestinal tract; CF: Cystic fibrosis; MRM: Multiple reaction monitoring.

1.3.4 Food residues in faeces

Studying food residues in faeces can reveal dietary components (Sistiaga et al. 2014) and provide information on the digestion of nutrients by animals, which is especially important for studying the dietary habits of some endangered species (Srivathsan et al. 2016) and wild animals (Bradley et al. 2007, De Barba et al. 2014). Most of these studies have been based on direct morphological examination of faeces (Moreno-Black 1978), stable isotope analysis (Blumenthal et al. 2012) and molecular methods such as specific DNA cloning and sequencing (Bradley et al. 2007). High-throughput sequencing simplified the operations and has been used in many studies. For example, researchers developed a method based on DNA metabarcoding multiplexing and next-generation sequencing to detect the composition of faeces (De Barba et al. 2014). Srivathsan et al. (2016) used metagenomics and metabarcoding in characterization of the primate faeces. Compared to genomic and metagenomic studies, few studies have used proteomics to study undigested food residues in faeces. Tsutaya et al. (2021) identified plant proteins in monkey faeces, and studied the taxonomy of the consumed food and investigated the dietary changes, showing breastfeeding and weaning patterns directly. With sufficient coverage of dietary databases, the improvement of faecal sample processing method and the reduction of experimental cost, research on dietary proteome in faeces is expected to increase.

1.3.5 Microorganisms in ruminant faeces

The microbiota plays an important role in animals, not only because it contributes to nutrient degradation, but also because it modulates animal metabolism and health (Andersen et al. 2021). A diverse GIT microbiome is expected to be more capable and resilient to changes compared to that with only a few species. Researchers reported that alpha-diversity increased as animals aged (Dill-McFarland et al. 2017) while some others found that animals tended to develop a more homogeneous and specific GIT microbiome during growth than after birth (Li et al. 2019). Characterization of the microbiota in ruminants helps to reveal the distribution and diversity in GIT, mechanisms of nutrient degradation and other functions, building a foundation for dietary manipulation, animal health and greenhouse gas control (Shi et al. 2014, Lopes et al. 2015). Ruminal microbiota has been explored extensively, although faecal microbiota is receiving increasing attention, more research is still needed, especially from the protein perspective.

1.3.5.1 Bacterial and archaeal compositions in ruminant faeces

Molecular biological approaches have been applied to study the microbiota: omics technologies such as metagenomics, metatranscriptomics and metaproteomics enabled in-depth reveal of the composition and functional patterns of the microbiota in ruminants. Bacteria generally dominate the rumen microbiome compared to archaea, fungi and viruses (Bainbridge et al. 2016). Researchers studied the bacterial microbiota of cattle and sheep by using gene sequencing and found that there were abundant *Firmicutes*, *Bacteroidetes* and *Proteobacteria* across the whole GIT (Mao et al. 2015, Wang et al. 2017). Faeces share many microbial communities with rumen, although faeces and rumen could be separated from each other based on the bacterial communities (Zeng et al. 2015, Holman and Gzyl 2019). The diversity of bacterial community was found to be significantly decreased as digesta passed through GIT (Frey et al. 2010, Mao et al. 2015), and the diet-induced changes in the rumen bacteria were reduced or eliminated in the faeces (Noel et al. 2019). Researchers (Tanca et al. 2017) found that *Firmicutes* and *Bacteroidetes* were dominant phyla in the faeces of sheep by using 16S rDNA sequencing, shotgun metagenomics combined with shotgun metaproteomics, with *Firmicutes* being the most abundant phylum, followed by *Bacteroidetes*. Similar results based on 16S sequences were reported in cattle, showing that *Firmicutes* dominated the faecal bacterial communities (Ozutsumi et al. 2005, Durso et al. 2010, Shanks et al. 2011, de Oliveira et al. 2013, Kim et al. 2014, Mao et al. 2015). However, this was in contrast to the rumen in some studies, where the most abundant phylum was found to be *Bacteroidetes* (Lee et al. 2012, Lopes et al. 2015). Within *Firmicutes*, *Ruminococcaceae*, *Lachnospiraceae* and *Clostridiaceae* were the most abundant families identified by metaproteomics in the sheep faeces and *Prevotellaceae* and *Bacteroidaceae* were the dominant families within *Bacteroidetes* (Tanca et al. 2017). In cattle faeces, *Ruminococcaceae* and *Lachnospiraceae* were also among the most dominant families of *Firmicutes, and Bacteroidaceae* was one of the dominant families within *Bacteroidetes* (de Oliveira et al. 2013, Kim et al. 2014). Genera such as *Clostridium*, *Bacteroides*, *Ruminococcus*, *Prevotella* and *Treponema* were abundant in the faeces of cattle (Dowd et al. 2008, Callaway et al. 2010, Mao et al. 2015). The relative abundances of *Clostridium* and *Bacteroides* in the rectum were higher than in other GIT regions (Mao et al. 2015, Zaheer et al. 2017), and they were also enriched in the cecum and colon of calves compared to the rumen, and increased proportionally in the cecum as the calves grew (Dias et al. 2018).

Methane in ruminants is primarily produced by methanogenic archaea in the rumen during fermentation (Noel et al. 2019), and is mostly emitted through eructation, which leads to an energy loss to animals at the same time (Hook et al. 2010). Many studies have mainly focused on the structure and function of archaeal communities in the rumen, although the lower GIT can be involved in methane production and emission as well (Murray et al. 1976, Hill et al. 2016, Bekele et al. 2022). In a study of sheep faeces (Tanca et al. 2017), researchers found that the archaeal *Euryarchaeota* was one of the most abundant microbial phyla based on the data of V4-16S rRNA, metagenomic and metaproteomic, and *Methanobacteriaceae* and *Methanocorpusculaceae* were found to be the dominant families within this phylum according to the metaproteomic results. Holman and Gzyl (2019) found that methanogenic genus *Methanobrevibacter*, which had been reported as the predominant archaeal genus in rumen (Janssen and Kirs 2008), was in all the 121 faecal samples of cattle from 52 studies, so was *Methanosphaera*, which had also been found to be one of the dominant methanogens in rumen (Hook et al. 2010, Cersosimo et al. 2016). Zhou et al. (2014) found that phylotypes close to *Methanobrevibacter* were the main taxonomy along the GIT of calves based on 16S rRNA sequencing and PCR-denaturing gradient gel electrophoresis. St-Pierre and Wright (2013) also reported that 16s rRNA gene sequences of *Methanobrevibacter* were the most frequently identified phylotypes in herbivores' gut. Differences between composition of ruminal and faecal communities have been noticed. For example, *Methanocorpusculum* was found in more than half of the 121 faecal samples but was almost absent from the 721 rumen samples of cattle (Holman and Gzyl 2019). Turnbull et al. (2012) also reported that *Methanocorpusculum* was usually found in hindgut rather than foregut of ruminants. Researchers found that *Methanocorpusculum labreanum*, *Methanocorpusculum sp. MSP* and *Methanoculleus bourgensis* only presented in the faeces of cattle compared to samples of rumen fluid (Daquiado et al. 2014). In their study (Daquiado et al. 2014), according to *mcr*A gene sequences, *Methanobrevibacter ruminantium* was proposed to be the predominant methanogenic archaea species in the barn floor manure, while the predominant methanogenic archaea species in rectal dung was *Methanocorpusculum labreanum*. *Methanobrevibacter smithii, Methanobrevibacter thaueri, Methanobrevibacter ruminantium* and *Methanobrevibacter millerae* were suggested to be core methanogen community of cows since they were found in each breed (Holstein, Jersey and Holstein-Jersey crossed) of the primiparous dairy cows at different days in milk time points (Cersosimo et al. 2016).

There are variations in composition and abundance of the microbial community between animal individuals, but bacterial communities can still be phylogenetically related (Brulc et al. 2009, Jami and Mizrahi 2012). The differences in microbial communities in ruminants have been found mainly attributable to diet (Shanks et al. 2011, Kim et al. 2014, Henderson et al. 2015), although age, breed, genetics and environment are also important influences (Henderson et al. 2015, Cersosimo et al. 2016, Dill-McFarland et al. 2017, Jiao et al. 2017, Noel et al. 2019).

1.3.5.2 Functions of ruminant faecal microbiota

Many polysaccharides from the diets of ruminants are not digestible by host enzymes, but they can be degraded by the bacteria throughout the GIT. *Prevotella*, *Clostridium*, *Ruminococcus* and *Bacteroides* have enzymes that break down structural carbohydrates to produce short-chain fatty acids such as acetate, propionate and formate, producing energy to animals, and are abundant in ruminant faeces (Flint et al. 2008, Holman and Gzyl 2019). Starch and sucrose metabolism is one of the core pathways of bacteria in ruminants, as is the metabolism of their hydrolytic products such as glucose, maltose and xylose (Wang et al. 2013, Li and Guan 2017). Bacteria play an important role in methane production since they produce the substrates such as acetate and formate for methanogenesis (Janssen and Kirs 2008, Noel et al. 2019). Methane can be formed by reduction of CO_2 or methanol by methanogens with hydrogen gas (Holman and Gzyl 2019, Matthews et al. 2019). Membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair and, energy metabolism were the five main pathways of the bacterial microbiota of cattle throughout the GIT predicted by metagenomics (Mao et al. 2015). Significant differences in abundance of bacterial gene families among GIT regions of the cattle were noted (Mao et al. 2015), for example, less digesta-associated microbiota of the cecum and colon was involved in carbohydrate metabolism and DNA replication and repair than those in the forestomach; and the abundance of mucosa-associated microbiota in the rectum were not as highly involved in amino acid metabolism as those in other parts of the GIT. A study of sheep faeces (Tanca et al. 2017) reported that the enriched microbial genes were related to membrane transport of molecules, DNA replication and repair, transcription, translation and protein folding; and the three most abundant potential metabolic pathways were glycolysis, L-arginine biosynthesis and peptidoglycan biosynthesis. Similar results were found in cattle faeces, in which the largest proportion of genes was related to carbohydrate and protein metabolism, based on metagenomics data (Durso et al. 2011).

Whereas genomics data could predict the potential functions and the involved pathways of the microbiota, proteomics results reveal the actual functions exerted by them. Based on metaproteomics results, researchers (Tanca et al. 2017) found that the main functions of the faecal microbiota in the sheep faeces were related to metabolism (especially carbohydrate degradation); protein synthesis and folding; and transport and signalling. Phylum-specific protein functions were noted in the sheep microbiota (Tanca et al. 2017), for example, class II fructose-BP aldolase, ABC transporter and bacterial flagellin as specific for *Firmicutes*, TonB-dependent receptor and ATPase C chain as specific for *Bacteroidetes*, and methanogenesis as specific for *Euryarchaeota*. Bacterial proteins in the sheep faeces (Tanca et al. 2017) were mainly involved in metabolic pathways such as polypeptide chain elongation, glycolysis and gluconeogenesis; there were also phylum-specific pathways, such as 1,2-propanediol degradation and butanoate metabolism as specific for *Firmicutes*, starch degradation for *Bacteroidetes*, and for *Euryarchaeota*, methanogenesis from CO_2 and methyl-coenzyme M reduction were among the 20 most relevant pathway-phylum combinations.

Correlations between microbiota in GIT of ruminants and nutrient degradation were expected to lay a foundation for improving dietary management and thus improving animal growth performance. Researchers (Lopes et al. 2015, Morgavi et al. 2015) found that the rumen fermentation and digestibility and, the host phenotype were influenced by the microbial diversity, and suggested the potential of metabolomics approach for monitoring biomarkers of the microbial functions. Shanks et al. (2011) found correlations between faecal starch concentration and relative abundance of *Firmicutes* and *Bacteroidetes*, respectively, and suggested the starch concentration to be a predictor of bacterial community structure. Changes in faecal microbial community of ruminants due to diseases such as subacute rumen acidosis and salmonellosis were studied (Mao et al. 2012, Munoz-Vargas et al. 2018), and faecal bacterial genes associated with antibiotic resistance and bacterial virulence were found (Durso et al. 2011), which might provide insights into potential microbial-based diagnostic or therapeutic targets. Methane emissions not only cause energy loss from animal production chains but also pollute the environment (Chang et al. 2019, Ugbogu et al. 2019, Bekele et al. 2022). Measurement approaches of methane emission, including using respiration and accumulation chambers and *in vitro* gas production techniques, have been widely used in ruminants (Storm et al. 2012, Hill et al. 2016, Bekele et al. 2022). Studies have developed models to predict the methane emission using dietary variables such as digestibility of hemicellulose, dry matter intake and

metabolizable energy intake (Ellis et al. 2007, Kebreab et al. 2008, Ramin and Huhtanen 2013). Researchers found that the animals which had lower residual feed intake could produce less methane (Hegarty et al. 2007). Sheep that emitted lower methane level were found to have smaller rumen than those high methane-emitting sheep (Matthews et al. 2019), which might also provide a basis for animal breeding to reduce methane emissions. The increases of diet digestibility and inclusion of dietary lipids were also found to decrease the methane production (Hristov et al. 2013). Dietary strategies, including using secondary plant metabolites (e.g., tannins and saponins) (Ku-Vera et al. 2020), seaweed and 3-nitrooxypropanol (Almeida et al. 2021), have been proposed for methane mitigation in ruminants.

1.4 Aims of the thesis

In conclusion, model fitting for retrospective performance analysis of animal growth has mostly made use of infrequent observations of BW, and the traditional method of quantifying the effects of interventions on growth is based on ADG estimates, ignoring the non-linear properties of the growth trajectories at different life stages. A new cereal grain preservation method using enzyme-catalysed urea was developed, but the effects of it on animal growth performance haven't been studied.

This thesis investigates the growth of cattle – firstly by investigation of mathematical models of growth, and then by the investigation of the effects of a recently developed method for cereal grain preservation using enzyme-catalysed urea. Finally, the thesis considers faecal proteomic examination as a potential tool to detect health and growth performance differences in cattle. The thesis had two main aims: (1) to find the most suitable growth models for cattle at different life stages using high-density bodyweight data and determine whether the best fitting model(s) improved parameter estimation in comparison with the traditional linear model; (2) to describe the effect of the enzyme-catalysed ammonia treatment of cereal grains on the growth performance of finishing beef cattle and their faecal proteomes.

Chapter 2 Growth Curve Models Using High-density Bodyweight Data for Accurate Parameter Estimation in Cattle

2.1 Introduction

The analysis of growth trajectories of animals is fundamental to many studies related to animal production, management, treatment and genetic selection (Berry et al. 2005, Crispim et al. 2015, Lupi et al. 2016). The non-linear trajectory of gain in bodyweight (BW) over the whole of life of animals is generally best represented by rate-state differential equations. Growth models such as the logistic (Verhulst 1838), Richards (Richards 1959), Gompertz (Gompertz 1825), Brody (Brody 1945) and von Bertalanffy models (Bertalanffy 1938) were derived from these equations, and have been widely used in studies to describe or predict the growth patterns of livestock (Lupi et al. 2015, Ghavi Hossein-Zadeh 2017, Selvaggi et al. 2017). However, model fitting for retrospective performance analysis of animal growth has mostly made use of relatively infrequent observations of BW, often on a monthly or quarterly basis (Moreira et al. 2016, Selvaggi et al. 2017), and sometimes even less frequently or simply at the beginning and end of a trial (Soberon et al. 2012). For example, researchers studied the major factors that could predict mature BW based on modelling only three BWs from the whole of life of each of 5,284 Angus cows (Goldberg and Racagnolo 2015); Crispim et al. (2015) identified and characterized single nucleotide polymorphisms (SNPs) associated with phenotypes (growth model parameters) in a multi-trait genome-wide association (GWAS) study of Brahman cattle, based on fitting growth models with only six BWs measured over the whole of life of 1,255 Brahman cattle.

Recent technical developments have enabled multiple records of BW to be made on every single animal every day, automatically weighing animals when they drink milk replacer or water, during milking or when walking from one pen or yard to another, as is becoming increasingly common on commercial farms (Gargiulo et al. 2018, Segerkvist et al. 2020). Researchers have estimated the energy balance (EB) of dairy cows by using high-density BW records, either alone or in combination with frequent body condition score (BCS) measurements (Thorup et al. 2012, Thorup et al. 2013). The use of daily BW records by

parametric or time series models has enabled more precise quantification of the performance of Nordic Red cows (Mäntysaari and Mäntysaari 2015), suggesting that high-density BWs might enable the development of more accurate models to describe the growth of cattle as they progress through distinct developmental phases.

In most practical applications, for convenient calculation, the traditional method of quantifying the effects of dietary interventions, disease, or other management interventions on livestock growth has been based on before-and-after measurements to generate linear slope or average daily gain (ADG) estimates (Reynolds et al. 1990, Lensink et al. 2000, Duthie et al. 2018). However, animal growth follows a complex, non-linear curve that can be divided into distinct phases: an initial, accelerating rate of gain is followed by an approximately linear phase, and finally by a decelerating rate of gain. Using suitable nonlinear models for growth of livestock at different life stages would be expected to improve the accuracy of parameter estimation compared to the traditional linear analyses, providing outcome measures for trials that more accurately reflect the effects of interventions. Any consequent improvement in precision of effect estimation should increase statistical power or allow studies with fewer animals but equivalent statistical power, thereby reducing cost and animal welfare imposts.

The overarching aim of this study was to determine the extent to which the application of non-linear models to high-density data might generate more precise estimates of growth parameters than the traditional linear model. Firstly, we aimed to find the best growth models for the whole of life of Holstein-Friesian cows for which high-density but intermittent BW data were available. Secondly, we wished to apply a similar approach to find the best models for Holstein and Holstein-cross calves using near-continuous BW observations from the first 100 days or so of life, and finally, to do the same for the last 100 days or so before slaughter in mixed-breed beef cattle.

2.2 Materials and methods

All data in this study were collected during the general animal husbandry management of these animals, no ethical approval was required.

2.2.1 Models and model comparison statistics

Figure 2-1 shows the workflow for the present study. To find the most suitable models for cattle growth, models (Table 2-1) were fitted to three data sets from different life stages: (1) whole of life (from birth to up to 4,077 days old); (2) early juvenile (up to 125 days); and (3) post-pubertal, sub-adult to adult (over 400 days old) by using the *nls* function in R, version 4.0.3 (R Core Team, 2020). Models were compared using the coefficient of determination ($R^2 = 1 - [\sum_{i=1}^{n}(Y_i - Y_i')^2 / \sum_{i=1}^{n}(Y_i - \bar{Y})^2]$, where Y_i is the *i*-th of *n* observations, Y_i' is the *i*-th model prediction, and $\bar{Y}$ is the mean of the observations), the Akaike information criterion ($AIC = 2p - 2\,ln(L)$; where *p* represents the number of parameters in the model, and *L* represents the maximum likelihood), and root-mean-square-error ($RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(Y_i - Y_i')^2}$) (Akaike 1974, Lupi et al. 2015, Burnham and Anderson 2016, Selvaggi et al. 2017). Within the same data set, a high R^2 for a model represents small differences between the observations and the model predictions, indicating that the model fits the data well. A low value of RMSE indicates better fit of the model. RMSE provides an absolute measure of fit (in the same units as the dependent variable) whereas R^2 represents a relative measure of fit. A lower AIC value indicates relatively higher model prediction accuracy. In addition, biological characteristics (birthweight and mature BW) estimated from the models were compared with the corresponding observations to assess the precision and plausibility of the estimates. Figures were made using *ggplot2* package (Wickham 2016) in R software.

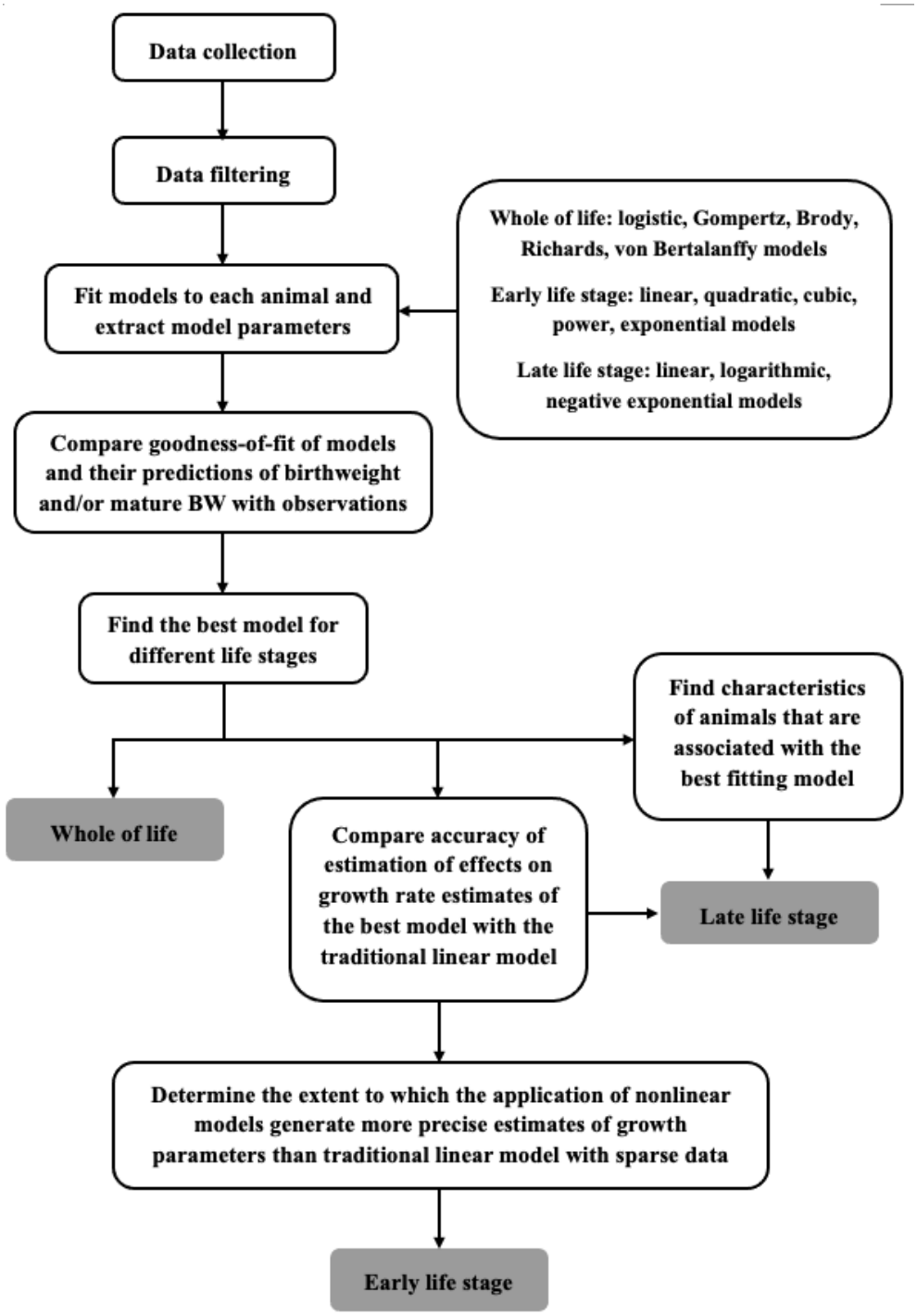


Figure 2-1. Flow diagram of the study.

Table 2-1. Models that were applied in the present study for fitting the bodyweights (cumulative BW) of cattle for whole of life, early and late life stages and their instantaneous growth rates.

Life stage	Model	Cumulative BW	Instantaneous growth rate	Parameters
Early and late life stages	Linear	$BW = at + b$	a	
Early life stage	Quadratic	$BW = at^2 + b$	$2at$	
	Cubic	$BW = at^3 + b$	$3at^2$	
	Power	$BW = t^a + b$	at^{a-1}	*t:* age in days
	Exponential	$BW = be^{at}$	abe^{at}	*a*: growth rate
Late life stage	Logarithmic	$BW = aln(t) + b$	a/t	*b*: initial weight
	Negative exponential	$BW = -be^{-at} + b$	Ake^{-kt}	*A*: mature BW
Whole of life	Gompertz	$BW = Ae^{-Be^{-kt}}$	$AkBe^{kt}/(e^{kt} + B)^2$	*B*: integration parameter
	Logistic	$BW = A(1 + Be^{-kt})^{-1}$	$AkBe^{-Be^{-kt}-kt}$	*k*: maturation rate
	Brody	$BW = A(1 - Be^{-kt})$	$AkBe^{-kt}$	*m*: inflection parameter
	Von Bertalanffy	$BW = A(1 - Be^{-kt})^3$	$3AkBe^{-kt}(1 - Be^{-kt})^2$	
	Richards	$BW = A(1 - Be^{-kt})^m$	$mAkBe^{-kt}(1 - Be^{-kt})^{m-1}$	

2.2.2 Whole of life data

There were 294,549 BW observations on 1,003 Holstein-Friesian cows from birth to up to the 8th lactation (mean ± standard deviation (SD): 1,400 ± 480 days old). The BW records during early life development (up to 515 ± 141 days) and the dry periods were intermittent, being measured approximately every 30 to 90 days. The BWs in lactation periods were automatically weighed two to four times daily on exit from the parlour, using a walk-over scales and a shedder to ensure an accurate weight for each individual cow. Data are owned by Scotland's Rural College (SRUC) and were made available to this project under a material transfer agreement (MTA).

We first detected and removed outliers, which might be caused by the equipment or movement of animals during measurements, by using linear regression to fit splines to the raw data. We only used the data from animals for which there were at least five BW observations from prior to the first lactation, and at least seven days of BW records during each lactation. Gestation is a complicating factor in bodyweight estimation of female animals: the combined weight of foetal membranes and foetal fluids, together with the developing foetus, will exceed 70 kg, even in small Jersey cattle (Eley et al. 1977). A further complication is the expected change in fatness of dairy cattle within lactations: ideally, cows in good body condition, with some fat cover, during the first weeks of lactation are expected to lose considerable BW (McCarthy et al. 2007), which is regained in later lactation (coincident in most cases with conception and gestation, occurring after d-45 of lactation). To minimize the dynamic effect of gestation and onset of lactation on bodyweight, and thus to develop a general growth model for the cows, we considered two distinct data subsets. Both subsets included the birthweights and all the observations before the onset of the 1st-lactation, none of which were from late pregnancy. In the first subset (three-bodyweight, TBW), three daily mean BWs from each cow in each lactation were included: the daily mean BWs for d-1, d-30 and d-60 of lactation (or the nearest day in the rare cases where that was not available). In the second reduced data set (lowest-bodyweight, LBW), the lightest within-lactation daily mean BW for each animal was taken for each lactation. The logistic, von Bertalanffy, Gompertz, Brody and Richards models were fitted to the data from each animal. The model performance was assessed according to R^2 and RMSE, model comparison was based on AIC (except for the Richards model that has four parameters, so AIC comparisons would not be valid with a 3-parameter model) as well as the accuracy of their estimations of birthweight and mature BW.

2.2.3 Early life stage data

174,072 near-continuous BW records were obtained from 399 mixed-breed calves (crossbred Aberdeen Angus, Holstein-Friesian, British Blue and Limousin), measured between February 2019 and February 2020. Calves were weighed manually within 24 h after birth and then weighed automatically by SRUC Crichton Royal Biocontrol units when the animals drank milk replacer or water from, at the earliest, the first day after birth (mean ± SD: 14 ± 11 days old) until, at the oldest, 126 days old (mean ± SD: 66 ± 24 days old). Data are owned by SRUC and were available to this project under a MTA.

We analysed the data of the calves that had a birthweight record and at least 14 days of BW observations. Outliers that might have been caused by the equipment or movement of animals during measurements were removed by using linear regression to fit splines to the raw data. To minimise fluctuations caused by variation in feed intake, gut or bladder fill, we calculated the daily mean BW for each calf from all of the records from any given day (excepting those filtered by spline fitting). The linear, quadratic, cubic, exponential and power models were fitted to the daily mean BW data and the model parameters R^2, AIC, RMSE and the precision of estimation of the birthweight by each of the models were compared to find the most suitable model for the early life stage.

Growth rate varies among breeds of cattle (Aviles et al. 2015) and between males and females (Marlowe and Gaines 1958, Daskiran et al. 2010), and we expected that the strength of these associations, and statistical power to detect them, would increase with increasing accuracy of growth rate parameter estimation. We compared the strength of association between these known correlates of growth rate (which in this analysis are analogous to competing interventions that might affect growth rate in a trial) and two alternative measures of growth rate: the growth rate parameter estimated from the best fitting growth model, and growth rate estimated from the traditional linear method (that is, change in weight between the beginning and end of a treatment period divided by number of days in the treatment period). A bootstrap analysis was conducted by re-sampling the BW data 1,000 times with replacement, and for each observation, fitting a generalized linear model (GLM) with breed and sex as independent variables, and the growth rate parameter estimate derived from either the linear or the non-linear model as the dependent variable. Because the competing models differed in their dependent variables, they could not be compared directly using RMSE or AIC. Instead, the strength of association between

the parameter estimates for the growth curves and the breed and sex was estimated using R^2, and the difference between R^2 estimates was considered to be the difference in performance of the competing growth rate estimation methods. For each bootstrapped data set, we calculated the difference between the R^2 value derived from the non-linear and the linear models, and for all observations we determined the mean difference, 95% confidence interval and a bootstrapped *p*-value to determine whether the observed differences differed significantly from zero ($p < 0.05$).

To quantify the effect of data density on statistical power, we repeated the bootstrapping analysis on six increasingly sparse data sets that corresponded with a notional reduction in frequency of measurement. Besides the full data set (birthweights were excluded), the reduced data sets contained data from the first measurement day, and data from every two days, four days, a week, two weeks, or four weeks. Animals that had at least three BWs in each reduced data set were retained. Model parameters were extracted and were used as described above by bootstrapping 1,000 times in models with breed and sex as independent variables, and either a linear or non-linear estimate as the dependent variable.

2.2.4 Late life stage data

The late life stage data consisted of 189,033 BW observations from 1,300 mixed-breed finishing beef cattle (Simmental, Charolais, Salers, Aberdeen Angus, Shorthorn, British Blue, Belgian Blue, Limousin and crossbreds). Cattle were weighed automatically when drinking, using the Beef Monitor system (Ritchie Agricultural, Forfar, Scotland) several times a day through the finishing (fattening) period of up to approximately 100 days before slaughter, between October 2016 and May 2018. Weighing errors were detected and removed using a Monte Carlo Markov chain (MCMC) method (unpublished). Data are owned by Harbro/Innovent/Scotbeef and were made available for this project.

For this study, we analysed the growth of animals with at least 60 days of BW data. Unlike the two previous data sets, in which the initial time point was birthdate, and starting weights (birthweights) were relatively similar, in this data set the age and weight of animals at the beginning of the period of observation varied greatly. Since the nutritional management during the finishing period is very distinct from that used prior to finishing, and in most cases, cattle growers will be interested in the rate of gain of animals from time of onset of feeding, in this analysis, the day of onset of feeding was taken as d-1, and the

age and weight of animals at d-1 were used as covariates for subsequent model fitting. The linear, negative exponential and logarithmic models were fitted to individual animal data. In the absence of an appropriate, independently measured mature weight, the model performance was compared using goodness-of-fit parameters only.

To determine whether specific characteristics of animals were associated with the best fitting model (the linear or the negative exponential model), we applied binary logistic regression in the generalized linear model function *glm* in R to test factors including age and BW at the beginning and end of the observation, the number of days on feed, weight gain during the trial, the farm, and breed (British or Continental). The effect of the factors was analysed by *Wald.test* (Bewick et al. 2005). Finally, the strength of association between the known correlates of growth rate (farms and/or breeds) and growth parameters derived from two alternative models of growth rate (the best non-linear model and the linear model) were compared using GLMs, using the same approach as described for calves above.

2.3 Results

2.3.1 Growth models for the whole of life of the cattle

After removing outliers and filtering, data from 906 animals remained: there were 19,070 BWs in the TBW data set, and 13,701 BWs in the LBW data set. The mean birthweight (± SD) of the 906 animals was 42.27 (± 5.41) kg and the mean heaviest BW ± SD was 635.28 ± 79.80 kg. In the TBW data set, the logistic, Gompertz and von Bertalanffy models were successfully fitted to all the animals individually, while the Brody model could not be fitted to two animals and the Richards model could not be fitted to 279 animals. Examples of each of these models as fitted to one animal from the TBW data set are shown in Figure 2-2 a. Bodyweights of all the animals were plotted in Figure 2-2 b, and the growth trajectories were drawn based on the mean values of the predicted parameters of each model for all the animals. The full results of model fitting are shown in Table 2-2 and Figure 2-3, including the mean value and standard deviation (SD) of the models' goodness-of-fit (R^2, AIC and RMSE) and estimated parameters, and the fitted BWs. Similar plots and results for animals in the LBW data set are in the Appendix (Figure 2-1 and 2-2, and Table 2-1).

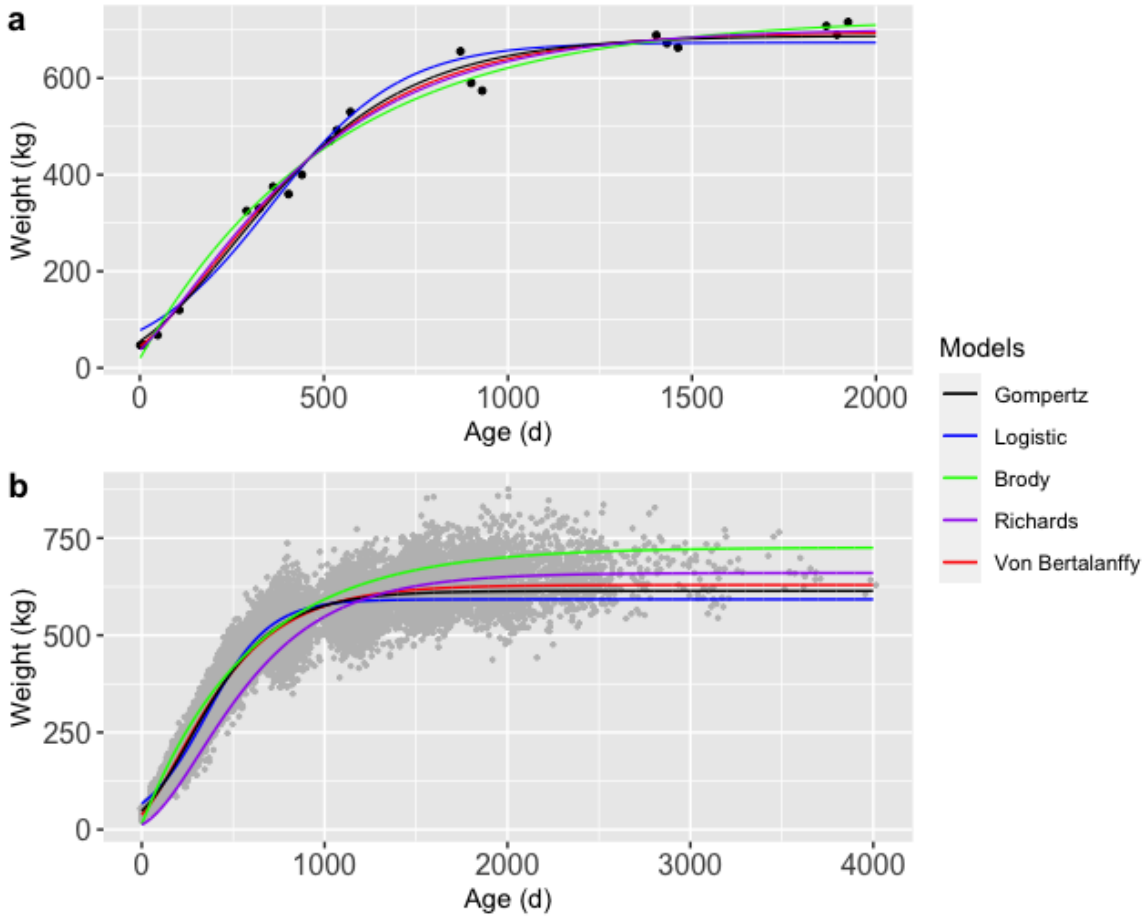
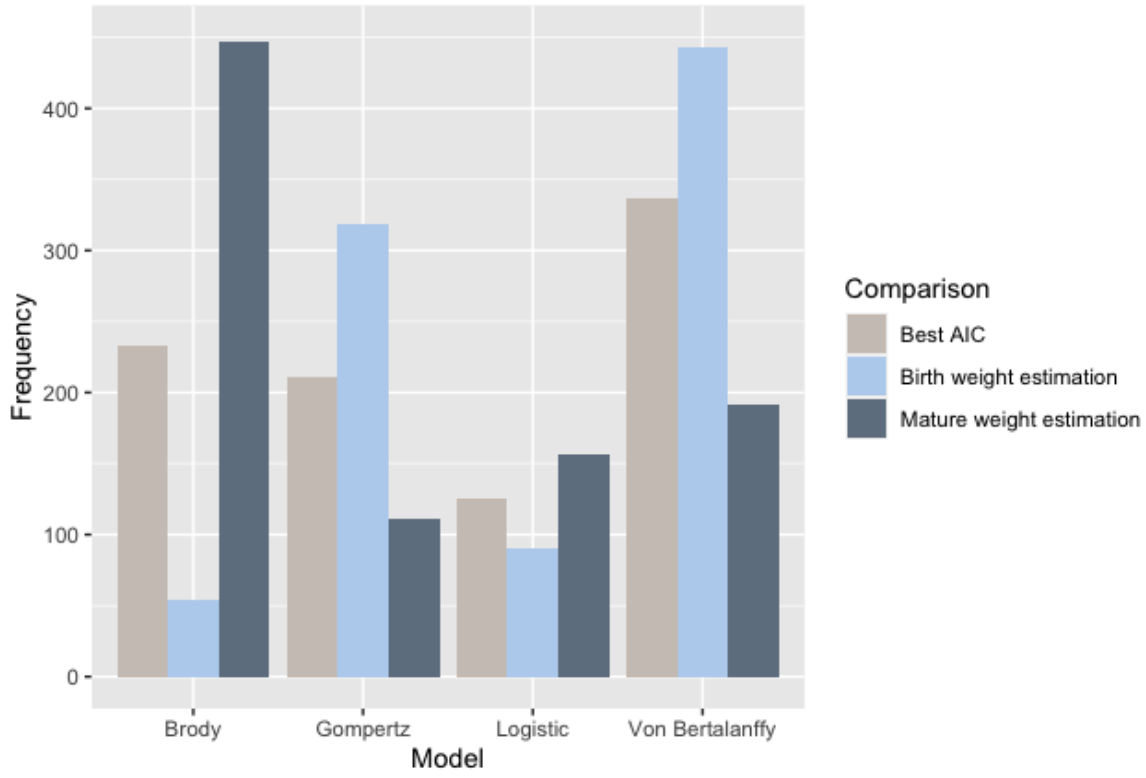


Figure 2-2. The whole of life bodyweights (BW) of cattle in the TBW data set (BW measurements used from each lactation were daily mean BWs for d-1, d-30 and d-60 of that lactation) and the predicted growth trajectories of models: (a) an example of fitting models to an individual cow (black dots are the BWs and the coloured lines are the predicted growth trajectories of models); (b) the grey dots are all the BWs from 906 cattle in this data set, and the coloured curves are the predicted growth trajectories from the models, for which the parameters were the mean values for each parameter and model for all of the cattle.

Table 2-2. The results of fitting models (failure cases excluded) to BWs of 906 cows from birth to a maximum of 4,000 days old in the TBW data set, in which the BWs in lactations were the daily mean BWs for d-1, d-30 and d-60 of that lactation.

Estimate		Growth model				
		Logistic	**Brody**	**Gompertz**	**Von Bertalanffy**	**Richards**
Number of successful models fitted		906/906	904/906	906/906	906/906	627/906
R^2		0.981 (0.013)	0.983 (0.011)	0.986 (0.0099)	0.987 (0.0095)	0.990 (0.0071)
AIC		204.43 (59.12)	200.70 (53.12)	196.35 (56.49)	194.84 (55.06)	201.47 (52.71)
RMSE		26.15 (9.43)	24.21 (7.68)	21.76 (8.01)	21.07 (7.59)	19.25 (6.76)
Parameter *A*		592.32 (62.42)	725.99 (210.65)	614.03 (62.74)	629.39 (66.94)	660.08 (92.51)
Parameter *B*		7.87 (1.87)	0.98 (0.02)	2.56 (0.28)	0.61 (0.05)	0.74 (0.23)
Parameter *k*		0.0058 (0.0013)	0.0017 (0.0004)	0.0037 (0.0007)	0.0030 (0.0006)	0.0025 (0.0008)
Estimated birthweight (kg)		74.76 (21.45)	-3.76 (29.211)	51.20 (18.11)	39.01 (17.48)	27.86 (11.54)
Birthweight difference	RMSE	38.85	54.27	20.04	17.59	18.27
(estimated-observed)	Mean Difference (SD)	+32.49 (21.31)	-46.02 (28.79)	+8.93 (17.95)	-3.26 (17.29)	-14.46 (11.19)
Mature BW difference	RMSE	51.19	201.68	46.93	55.21	132.86
(estimated-observed)	Mean Difference (SD)	-40.33 (31.54)	+70.44 (189.09)	-20.18 (42.40)	-6.45 (54.86)	+16.68 (131.93)

Results are presented as mean (standard deviation) unless indicated otherwise. Parameter *A*, *B* and *k* are parameters in the models, represent mature BW, integration parameter and maturation rate, respectively. RMSE of birthweight or mature BW: Square root of the mean of the differences between estimated birthweights/mature BWs of models and the corresponding observations of all the animals. Mean (SD) of birthweight or mature BW estimation: mean value and standard deviation of the differences between the estimated birthweights/mature BWs and the actual observations of all the animals, '+' means over-estimated, '-' means under-estimated.

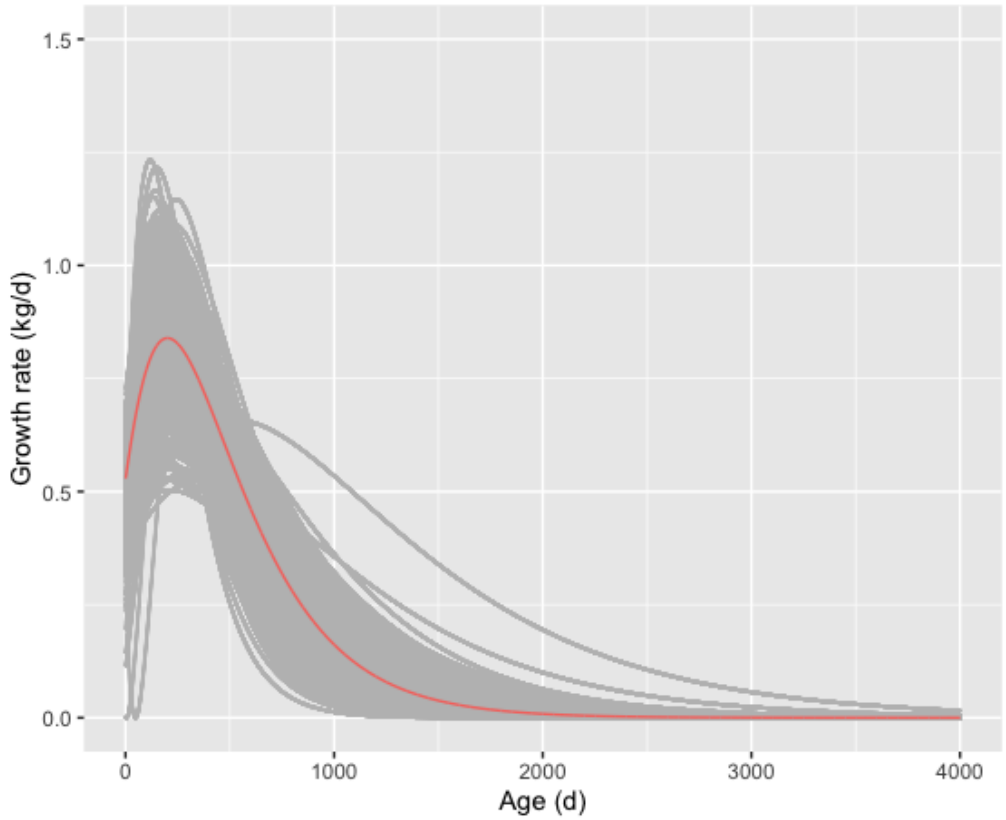


Each column represents the number of cows for which that model provided the best fit or the best estimation.

Figure 2-3. Model comparison (the Richards model was excluded) according to the best AIC and estimations of birthweight and mature BW for each individual cow in the TBW data set, in which the data in lactations were the daily mean BWs of d-1, d-30 and d-60 of that lactation.

With the exception of the Richards model, all the growth models fitted quite well to the growth data of all the cattle. The R^2 values of the models were all above 0.981, and the mean RMSE values were no more than 26.15 kg. The average AIC values of all the models ranged from 194.84 to 204.43. The von Bertalanffy model was the best model for 287 cows according to the lowest AIC values, followed by the Gompertz model (211 cows), the Brody model (199 cows) and the logistic model (125 cows). The Richards model provided 84 cows with low AIC, however, it failed to fit with the growth of many cattle in our case (279 cows), so it was not considered further as a candidate for best model. Among the 211 cows for which the Gompertz model fitted best, the AIC of 117 were not significantly better (difference in AIC < 2) than the Von Bertalanffy model. Consequently, the Von Bertalanffy model was the best model for the animals according to the model statistics.

The von Bertalanffy model provided the closest estimates of birthweight to the observations of most of the cattle (443 cows), followed by the Gompertz model (319 cows), logistic model (90 cows) and the Brody model (54 cows). The RMSE of the birthweight estimation of the von Bertalanffy model was the lowest (17.59 kg), followed by the Gompertz model (20.04 kg). The average difference between the birthweight estimates of the Von Bertalanffy model and the observed birthweights was the smallest (underestimated by 3.26 kg). The estimated mature BW from the Brody model were the closest to the observed maximal weights of most of the cattle (447 cows), followed by the von Bertalanffy model (191 cows), the logistic model (157 cows) and the Gompertz model (111 cows). However, the RMSE and the average difference between the estimates of the Brody model and the observed maximal weights were the largest among the models. Although the von Bertalanffy model ranked second in mature BW estimation, it provided close agreement of mature BW, and it provided the smallest average differences between the estimates and the observations (on average, underestimated by 6.45 kg). Consequently, we considered that the von Bertalanffy model was the best model for fitting the whole of life growth curves of the Holstein-Friesian cows due to the best goodness-of-fit and accurate estimation of BWs. The estimated growth rates (kg/d) for the whole of life of the cattle using the von Bertalanffy model are shown in Figure 2-4. When the animals were 203 ± 37 days old, they reached a maximum growth rate at 0.84 ± 0.12 kg/d.



The grey lines and the red line represent individual cattle and the mean of all cattle, respectively.

Figure 2-4. The predicted growth rates (from birth to 4,000 days old) of the 906 cows in the TBW data set (the BWs in lactations were the daily mean BWs for d-1, d-30 and d-60 of that lactation) using the von Bertalanffy model.

2.3.2 Growth modelling of calves

After cleaning up the data and calculating the daily mean BW of each calf, there were 20,846 data points from 361 calves. There were 15 crossbred Aberdeen Angus (AAX) bulls and 8 heifers; 4 crossbred Belgian Blue (BBX) bulls and 4 heifers; 98 Holstein-Friesian (Holstein) bulls and 171 heifers; 35 Limousin (LIMO) bulls and 26 heifers. Each calf had a birthweight and at least 15 BWs, including BWs from at the earliest, 2 days old (8 ± 13 d) to the oldest at 125 days old (70 ± 21 d). Based on the model fitting results of all the individual calves, mean values of parameters of each model for 361 calves were calculated. The growth trajectories from birth to 125 days old predicted by each model can be found in Figure 2-5. Goodness-of-fit and results of birthweight estimation of each model are in Table 2-3 and Figure 2-6.

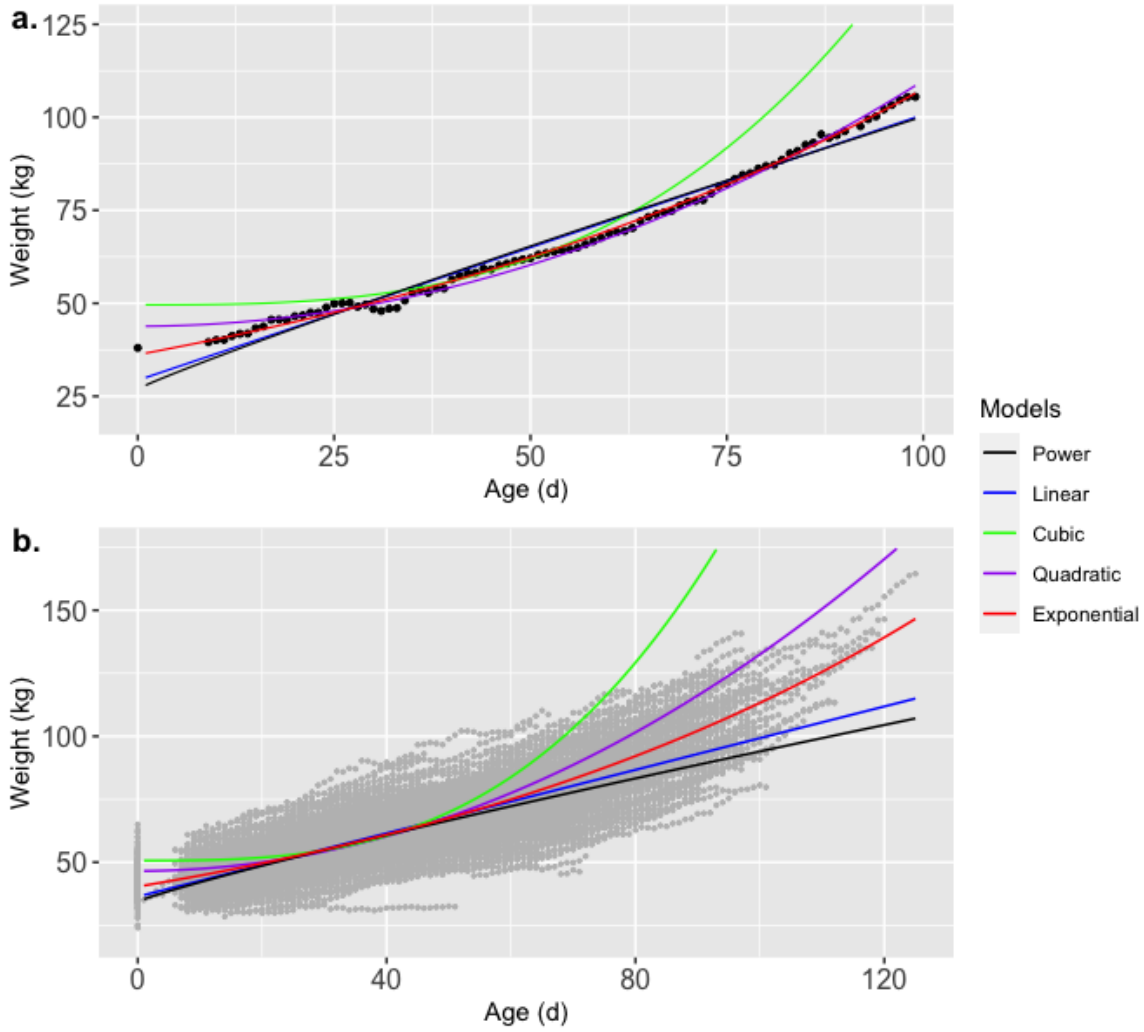
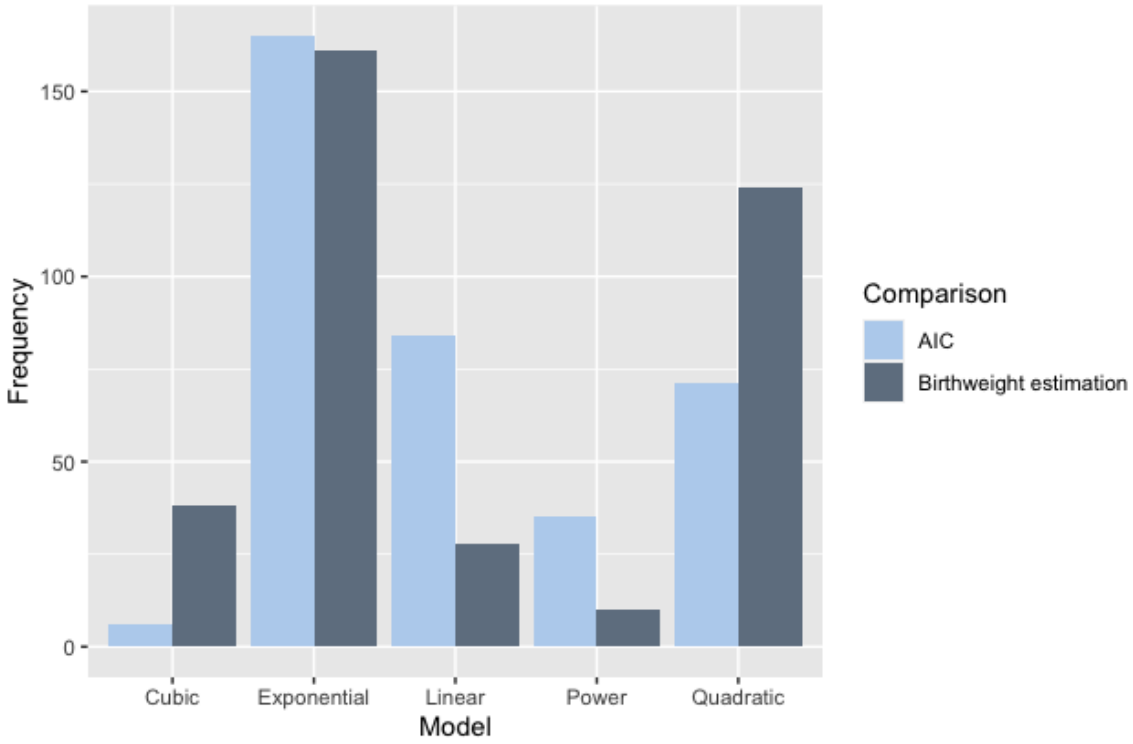


Figure 2-5. The bodyweights (BWs) of cattle in the early stage of life (from birth to 125 days old) and the fitted growth trajectories of models: (a) an example of fitting models to an individual calf (black dots are the observations and the coloured lines are the fitted trajectories); (b) the grey dots are the BWs of all the 361 calves and the coloured curves are the fitted growth trajectories of models, of which the parameters were the mean values of all the cattle.

Table 2-3. The overall results of fitting models to the growth of 361 calves in the early stage of life (from birth to 125 days old).

Estimate		Growth model				
		Linear	**Quadratic**	**Cubic**	**Power**	**Exponential**
R^2		0.947 (0.07)	0.935 (0.08)	0.871 (0.09)	0.937 (0.08)	0.954 (0.07)
RMSE		2.10 (0.90)	2.34 (0.98)	3.69 (1.57)	2.27 (0.91)	1.79 (0.68)
AIC		255.76 (125.45)	268.35 (125.87)	323.48 (154.47)	265.32 (126.52)	235.57 (105.83)
Parameter *a*		0.63 (0.17)	0.0086 (0.0037)	0.00015 (0.00012)	0.89 (0.076)	0.010 (0.0024)
Parameter *b*		36.37 (7.21)	46.55 (6.50)	50.66 (7.08)	34.35 (7.30)	40.34 (6.01)
Birthweight difference	RMSE	9.12	6.25	10.09	10.84	4.96
(estimated-observed)	Mean (SD)	-7.63 (4.97)	+4.04 (4.78)	+8.26 (5.81)	-9.80 (4.65)	-2.81 (4.10)

Results are mean (standard deviation) unless indicated otherwise. Parameter *a* and *b* are parameters in models, represent growth rate and birthweight, respectively. RMSE of birthweight: square root of the mean of the differences between estimated birthweights of models and the birthweights of all the animals. Mean (SD) of birthweight estimation: mean value and standard deviation of the differences between the estimated birthweights and the actual observations of all the animals, '+' means over-estimated, '-' means under-estimated.



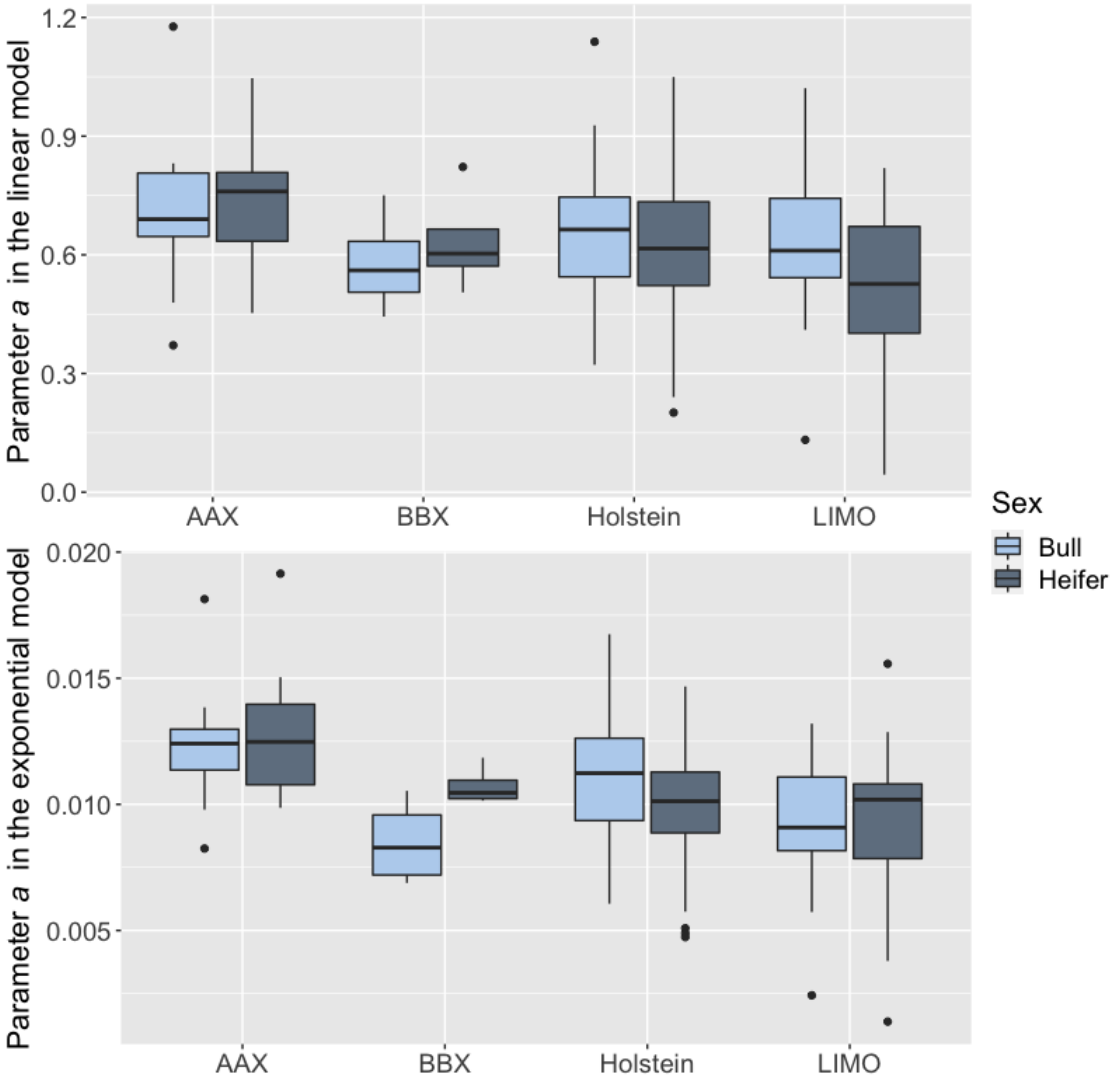
Each column represents the number of calves for which the model provided the best fit or estimation.

Figure 2-6. Model comparison according to the best AIC and estimations of birthweight for the 361 calves from birth to 125 days old.

Each of the five models provided a good fit for the growth of calves, with high R^2 values for each calf. The mean R^2 for the models for 361 calves were all above 0.87. The exponential model was the best model for 165 calves according to the lowest AIC values, followed by the linear model (84 calves), the quadratic model (71 calves), the power model (35 calves) and the cubic model (6 calves). Among the 165 calves that fitted the best with the exponential model, the model provided 155 calves with significantly lower AIC (difference in AIC > 2) than the linear model, which was the second best model; among the 84 calves that fitted the best with the linear model, AIC values of 11 of them were not significantly different from the exponential model. According to model statistics, the exponential model is the best model for the growth of cattle in the early life stage. The exponential model also provided the most accurate estimates of birthweight for 162 of 361 calves, followed by the quadratic model (124 calves), the cubic model (38 calves), the linear model (27 calves) and the power model (10 calves). The exponential model mostly under-estimated the birthweights (266 in 361 calves), while the quadratic model mostly over-estimated the birthweights (291 in

361 calves). The average birthweight estimated by the exponential model was 40.09 ± 6.06 kg, and the quadratic model was 46.94 ± 6.73 kg. Overall, the exponential model provided both the best model statistics and birthweight estimation of 95 calves at the same time, while the quadratic model only provided 42 calves with the best model statistics and birthweight estimation at the same time, and for the linear model, only 12. Consequently, the exponential model was proposed to be the best model for the growth of cattle at early life stages (from birth to 125 days old). The growth rate trajectories of all the calves predicted by the exponential model are shown in Appendix (Figure 2-3).

When models were constructed to quantify the effect of breed, sex and their interaction (Figure 2-7), the exponential model was more sensitive to differences in these independent variables, as the *p*-values for the effects of breed, sex and their interaction for the estimation of parameter *a* were 6.21×10^{-8}, 0.0030 and 0.035, respectively, in contrast to the *p*-values for the effects of breed and sex on parameter *a* in the linear model, being only 0.0087 and 0.074 with a non-significant interaction ($p = 0.15$). The exponential model provided 13.7 % R^2 estimate for breed, sex and their interaction, while R^2 of the model for the linear model parameter *a* was 5.5 %. It showed that there were 8.2 percentage points (pp) difference (95% CI: 3.2 - 13.3 pp, $p < 0.01$) between the exponential model estimates and the linear model estimates by bootstrapping 1,000 samples.



AAX: Aberdeen Angus, 15 bulls and 8 heifers; BBX: Belgian Blue, 4 bulls and 4 heifers; Holstein: Holstein-Friesian, 98 bulls and 171 heifers; LIMO: Limousin, 35 bulls and 26 heifers.

Figure 2-7. Effects of breed and sex on the parameter *a* in (above) the linear model and (below) the exponential model (the most suitable model).

Data sets were made to test the effect of reducing the density of the data on the statistical power of the exponential model. The reduced data sets included the data thinned to every two days, every four days, weekly, every two weeks, and every four weeks. Only 183 of the 361 calves yielded at least three measurements when thinned to 28-day frequency. Therefore, to make the reduced data sets comparable across different degrees of thinning, only these 183 animals were used in this analysis. The overview of the number of BW data-points per animal in each data set is shown in Table 2-4. Among the 183 calves, there were one AAX bull and one heifer, two BBX bulls and three heifers, 13 LIMO bulls and 6 heifers and 28 Holstein bulls and 129 heifers. Due to the limited number of animals in some breeds, animals except for Holstein were considered as a group - beef cattle. The estimation of effects of breed and sex on parameter estimates using each data set is shown below (Table 2-4). It confirmed that the exponential model was more sensitive to the differences in the independent variables, showing significant effects of breed on the growth parameters while the linear model showed nonsignificant effects of it. The interaction effects of breed and sex on growth rate estimates of the exponential model were significant until the data frequency decreased to every 14 days, while the linear model always showed it nonsignificant. In figure 2-8, R^2 estimates of the models with parameter estimates as dependent variables, and breed and sex as independent variable varied as the frequency of BW measurement decreased. The exponential model always generated much higher R^2 estimates than those provided by the traditional linear model. However, the R^2 estimates of both the linear model and the exponential model did not decrease as the data frequency decreased, and even slightly increased when the data were thinned to every 14 days or 28 days.

Table 2-4. The effects of breed and sex on parameter estimates in the linear model and exponential model of the 183 calves, for which the reduced data sets included the data filtered to once every two days, once every four days, once every seven days, once every 14 days and once every 28 days.

Frequency (day)	Observations / calf				Model	*P* - value		
	Min	Max	Med	Mean		Breed	Sex	Breed × Sex
1	57	115	73	75	Linear	0.51	0.50	0.76
					Exponential	0.00076	0.31	0.033
2	29	58	37	38	Linear	0.47	0.51	0.77
					Exponential	0.00054	0.32	0.034
4	15	29	19	19	Linear	0.49	0.47	0.83
					Exponential	0.00044	0.37	0.042
7	9	17	11	11	Linear	0.52	0.41	0.87
					Exponential	0.0004	0.34	0.058
14	5	9	6	6	Linear	0.43	0.45	0.99
					Exponential	< 0.001	0.31	0.13
28	3	5	3	3	Linear	0.25	0.61	0.93
					Exponential	< 0.001	0.37	0.19

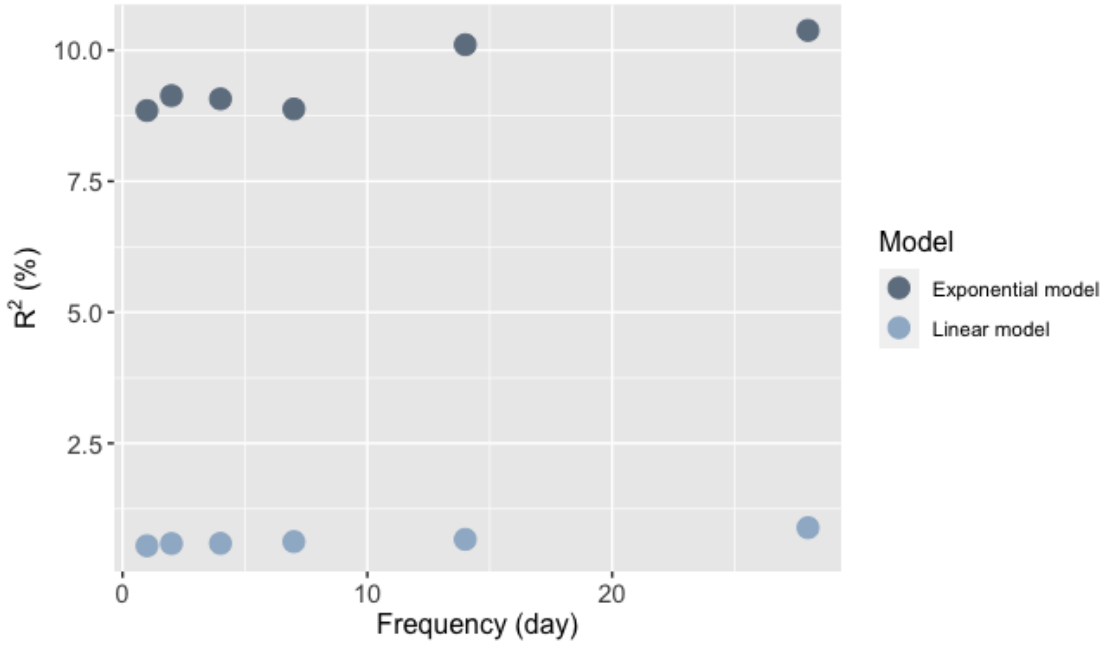


Figure 2-8. The R^2 estimates provided by the linear model and the exponential model to the effects of breed and sex, using the reduced data sets that included the data of the 183 calves filtered to once every two days, once every four days, once every seven days, once every 14 days and once every 28 days.

2.3.3 Growth model for the finishing period of beef cattle

After removing outliers and incomplete cases, 22,936 BW observations from 268 fattening cattle with BW records of no less than 60 days were retained. The day of onset of feeding was taken as d-1, and the age and weight of animals at d-1 were used as covariates for subsequent model fitting. Linear, negative exponential and logarithmic models were fitted to the data for each animal. However, unlike the linear model (R^2 = 0.946 ± 0.050) and the logarithmic model (R^2 = 0.788 ± 0.057), which could be fitted to the BWs of all the animals, the negative exponential model could be fitted only to the BW data from 152 animals (R^2 = 0.951 ± 0.038). According to the comparisons of model statistics, the linear model fitted the best (lowest AIC, R^2 = 0.958 ± 0.030) for 171 finishing beef cattle (64% of 268 cattle) while the negative exponential model fitted the best (lowest AIC, R^2 = 0.949 ± 0.045) for 97 finishing beef cattle (36% of 268 cattle), the logarithmic model never provided a good fit to the growth curves of cattle over the finishing period. Among the 152 animals that could be fitted with all the three models, 22 of them could be fitted well with both the linear model and the negative exponential model due to the close values of R^2 (difference in R^2 < 0.001) and AIC

(difference in AIC < 2). Figure 2-9 shows two examples of fitting models to finishing beef cattle and all cleaned BWs of the 268 finishing beef cattle.

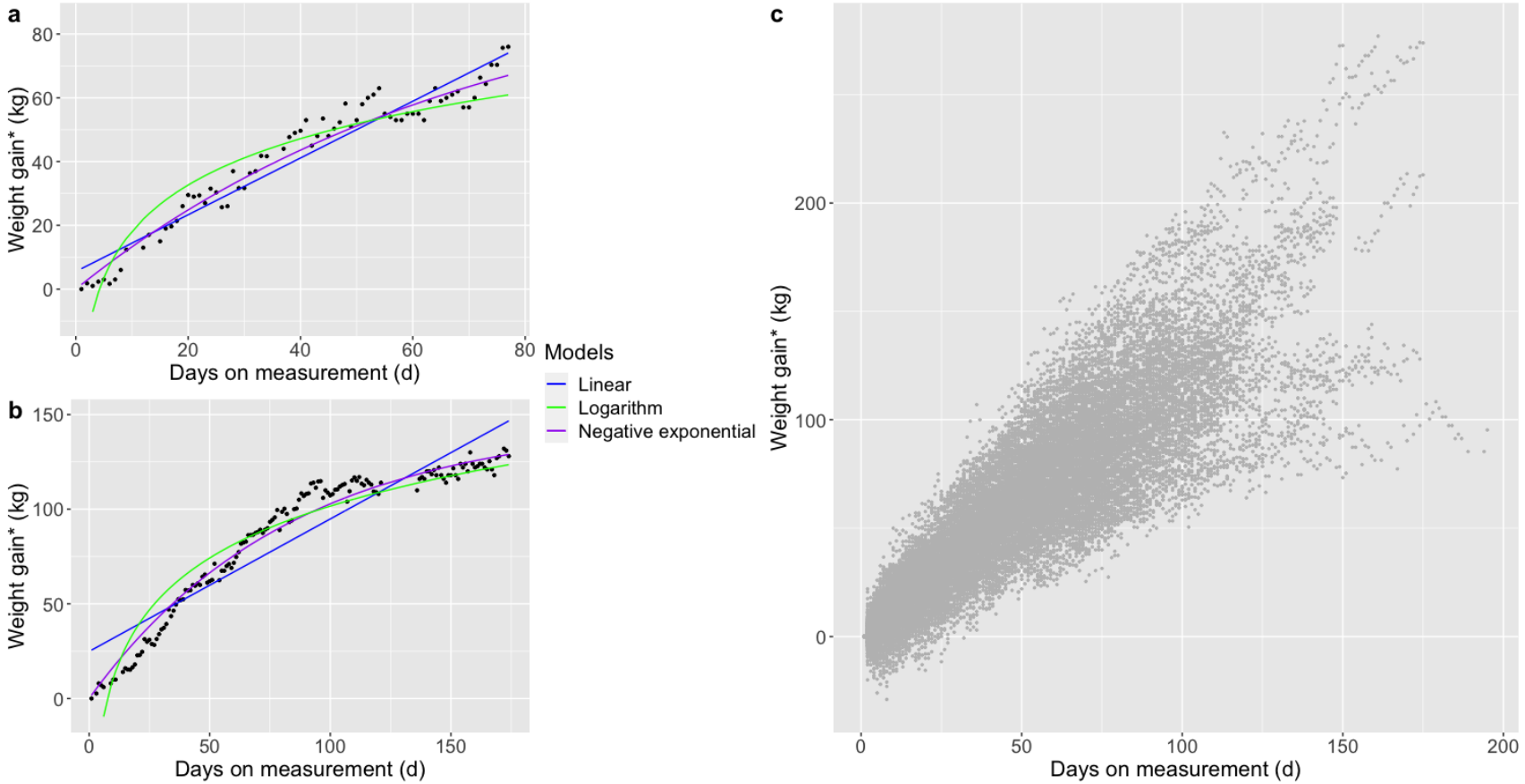
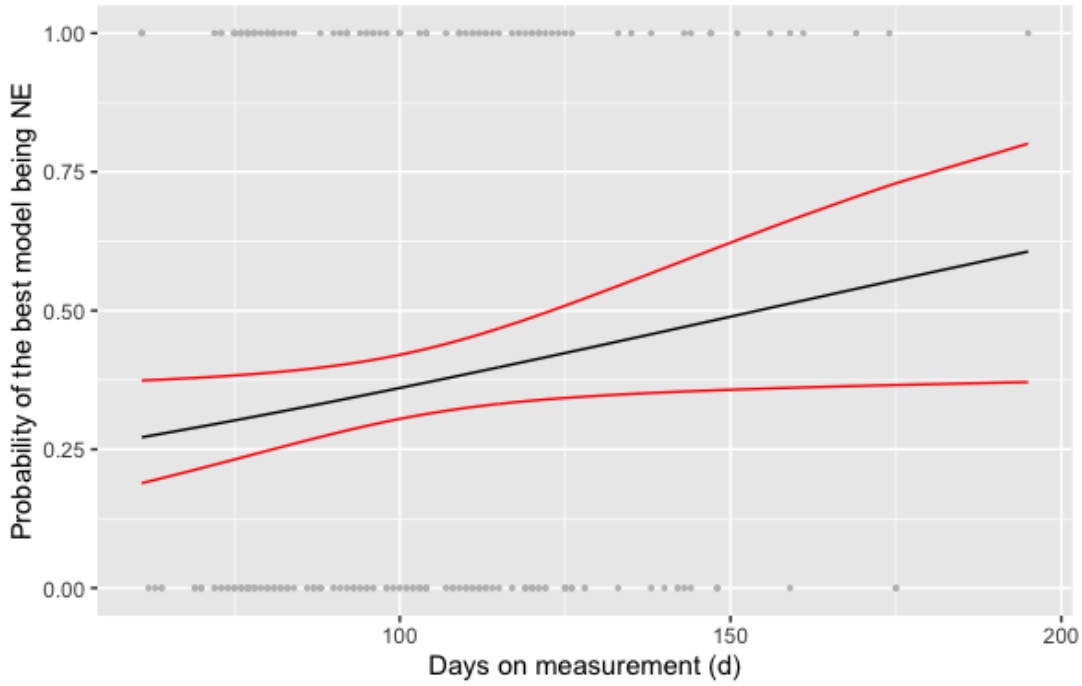


Figure 2-9. The weight gains of finishing beef cattle in the late stage of life and the fitted growth trajectories of models: (a) and (b) are two examples (black dots are the weight gains and the coloured lines are the fitted trajectories), (a) shows that the linear model is the best model and (b) shows that the negative exponential model is the best; (c) shows all the cleaned data of the 268 finishing beef cattle.

Because we expected that some animals in this phase of development would have been approaching their maximum mature BW, we tested whether the age of animals or their BWs would influence whether the linear model or the negative exponential model was better. Among the 268 finishing beef cattle, the birth dates of 160 animals were known. For these cattle, we gathered the data including their age at the beginning (580 ± 107 days old) and at the end of the measurement (687 ± 112 days old), their duration on feed (107 ± 26 days), their weight gains during the observation period (123 ± 35 kg), their BWs at the beginning (550 ± 77 kg) and at the end of the measurement (673 ± 68 kg) and farm information (3 farms). These variables were used as independent variables in a binary logistic regression model in which the outcome variable was whether the linear model or the negative exponential model performed the best. Only the number of days that the cattle were on feed significantly affected ($p = 0.041$) the choice of best model for 160 cattle. The potential effect was then tested on all the 268 cattle (mean ± SD of days on feed were 100 ± 25 d). The result confirmed that the days of the cattle on feed significantly influenced ($p = 0.036$) the choice of best model. The probability of the best model being the negative exponential model is plotted against days on measurement below (Figure 2-10). It shows that the longer an animal is on feed, the more likely it is that the best model will be the negative exponential model. In the present study, there were 88 cattle whose breeds were known; these were divided into British (9 cattle) and Continental (79 cattle). There were no significant effects of the breeds ($p = 0.99$) on the model comparison results: the growth of all the British cattle fitted better with the linear model, while the growth of nearly half of Continental cattle (40) fitted better with the linear model and the rest fitted better with the negative exponential model (39). The days on feed of the British cattle varied from 69 to 175 d, for Continental cattle it varied from 70 to 195 d. The effect of days on feed on model selection was confirmed in this small subset as well, showing the *p*-value as 0.034. In conclusion, the longer the cattle were on feed, the more likely that the best model for growth would be the negative exponential model.



NE: the negative exponential model. The grey dots (jitter at top and bottom of panel) were the results of model comparison of the 268 cattle: dots on the lower x-axis are the days on feed for which the best model was the linear model, and those on the top horizontal x-axis are the days on feed for which the best model was the negative exponential model. The area between the two red lines represents the 95 % CI of the probability.

Figure 2-10. The probability (black line) of the best model being the negative exponential model over the days of cattle on feed (measurement).

For the 152 finishing beef cattle (days on feed, mean ± sd = 103 ± 27 d) for which data could be fitted with the negative exponential models, the comparison of precision of parameter estimation by the traditional linear model and by the best nonlinear model was done similar to the study on calves, as described above. Farm information of all these animals and breeds of 53 of them were known, GLMs were firstly fitted with farm (two farms) and breed (Continental or British) of the 53 animals as independent variables, and the growth rate parameter estimates as the dependent variable. It showed that breed did not significantly affect the parameter estimates in either model, but there was a significant effect of farm on growth rate estimates in the linear model (p = 0.00079), but no significant effect of farm on growth rate estimates in the negative exponential model (p = 0.86). Farm was then used as the independent variable in testing all the 152 animals, which confirmed the better performance of the linear model: p-values of farm effect on growth rate estimates were 0.0031 and 1.0×10^{-7} of the negative exponential model and the linear model, respectively. Bootstrapping 1,000 samples showed that the negative exponential model

provided R^2 estimates that were around 14 percentage points (pp) lower than the linear model estimates (12 vs 26 %).

In conclusion, the best models for the growth of beef cattle during the fattening phase of life were the linear model and the negative exponential model, in many cases the linear model was easier than the negative exponential model to be fitted with the growth of the cattle. The linear model provided the best goodness-of-fit of fitting the growth of more animals compared to the other models, however, the longer the cattle were on feed the more likely that the best model for growth would be the negative exponential model. For those animals for which data was available for fitting GLMs, the linear model was more sensitive to the effects and provided overall more precise parameter estimates than the negative exponential model. The finding suggested that to evaluate the best model for the growth of animals before analysing effects of interventions might be useful.

2.4 Discussion

Our analysis with high-density, but non-continuous data suggested that the von Bertalanffy model was the best model (compared with the Brody, Gompertz, logistic and Richards models) to describe the whole of life growth trajectories of the lactating Holstein-Friesian cows. For near-continuous BW observations of Holstein and Holstein-cross dairy calves up to 125 d, the exponential model performed the best. Applying this model improved the precision of the estimates of the effects of breed and sex, providing greater statistical power than the traditional linear model. The best models for the growth of beef cattle during the fattening phase of life were the linear model and the negative exponential model, with the negative exponential model becoming the preferred model for cattle that stayed longer in the fattening phase, although the linear models provided better effect estimates for independent variables that were expected to influence growth rate. These two findings together are consistent with the theory underlying the growth models fitted to the growth of whole of life of cows. The growth of animals in different phases could be analysed separately using the most suitable models.

The von Bertalanffy model fitted the best to the growth of whole of life of cows in the present study, with not only high goodness-of-fit but also accurate biological estimates. Consistent with our findings, Berry et.al (2005) reported that the von Bertalanffy model performed the best among the Brody, logistic, Richards and Gompertz models in modelling the growth of three strains of Holstein-Friesian female dairy cattle using weekly BWs. Vaccaro and Rivero (1985) also used the von Bertalanffy model for analysis of growth of Holstein-Friesian cows in the Venezuelan tropics. It was also reported to be the best model to describe the growth of other cattle breeds such as Zebu cattle and Nelore cattle (Lopes et al. 2012) and has been used in Chianina and Nelore cattle as well to compare growth parameters (Carrijo and Duarte 1999). However, it showed poor goodness-of-fit compared to the Brody and Richards models in synthetic breeds of Charolais, Angus and Galloway female beef cattle (Goonewardene et al. 1981). For these beef cattle and also for purebred Hereford, Goonewardene et al. (1981) found that the Brody model was the most suitable growth model according to high goodness-of-fit and accurate prediction of BWs. Forni et al. (2009) reported that the Brody model was the best model for Nelore female beef cattle, providing accurate birthweights compared to the Gompertz and von Bertalanffy models. However, in the present study, it indeed provided lots of Holstein-Friesian cows with accurate mature BW estimates but also provided the worst estimations of birthweight

among the growth models. The Richards model is an empirical function with one more parameter than the other models. It was found to give better prediction of BWs of Angus pasture-fed cows from birth to maturity (Goldberg and Racagnolo 2015) and more precise prediction of mature BWs of Podolica bulls than the other models (Selvaggi et al. 2017). However, at the same time as improving model fit, the Richards model increases the probability of overfitting and numerical difficulties in growth modelling. Consistent with the present study, researchers failed to fit it to the growth of Brahman cattle (Crispim et al. 2015). Two more models were tested in the present study: the logistic and Gompertz model. The former was initially used for modelling growth of human population and the latter was developed for human mortality. Although the goodness-of-fit of the two models were not bad, they provided very poor biological estimates for the Holstein-Friesian cows in the present study. Both models have been reported to perform well in fitting to the growth of small livestock such as sheep (Lupi et al. 2015, Ghavi Hossein-Zadeh 2017), and birds such as domestic pigeon (Gao et al. 2016) and meat quail (Gotuzzo et al. 2019). The decision of which is the most suitable growth model depends on many factors such as species, breeds, sex, changes in environmental factors, differences in feeding management and results of genetic selection (Brown et al. 1976, Selvaggi et al. 2017). Comparison between models should not only focus on purely statistical criteria, but also be based on practical biological characteristics. As noted previously (Brown et al. 1976), the selection of the models and their modified forms should depend upon the nature of the study and the intended application of the results. With the accurate analysis of whole of life growth trajectories of cattle, heritability of model parameters and their genetic correlations can be estimated, selection of high-performance animals and the most suitable time for slaughter might be chosen (Luo et al. 2015, Lupi et al. 2016, Ghavi Hossein-Zadeh 2017).

The exponential model was the best model to describe the growth of calves up to 125 d compared with the linear, quadratic, power and cubic models. Few studies have examined growth models of cattle in their early life stages. In 1995, some researchers found that the linear model was the most suitable growth model for Retinta beef cattle up to the weaning according to the best goodness-of-fit, compared with the logistic, Gompertz, Richards, Brody and von Bertalanffy models, polynomial model with up to fourth degree and diverse exponential models (Berlanga et al. 1995). In a recent study (Quigley et al. 2021), researchers measured BWs of calves weekly up to 64 days old and every four weeks for calves over 60 days old and below 114 days old, and generated a quadratic correlation between ages and BWs. However, although the quadratic model ranked second in

estimation of birthweights of calves in the present study, the exponential model provided better goodness-of-fit and more precise birthweight estimation.

Historically, where researchers have had access to several weight measurements, as well as the traditional before-and-after measurement to evaluate animal growth rates, the bodyweight data has sometimes been split into two or more shorter time periods to minimize the errors. For example, Donovan et al. (1998) split the growth from birth to 14 months into two periods (birth to 6 months and 6 months to 14 months) to study the effects of colostrum-dependent immune status and disease conditions on heifer growth. Malhado (2013) calculated ADG of cattle from birth to 205 days old and from 205 to 365 days old to study the influence of inbreeding depression on cattle growth. According to the findings in the present study, application of the exponential model would be expected to improve accuracy of description of animal growth patterns, even with as few as four datapoints.

The exponential model for calves provided more precise parameter estimates than the traditional linear model, and it showed higher sensitivity to the effects of breed and sex compared to that in the traditional method, which remains widely used in practice (Reynolds et al. 1990, Lensink et al. 2000, Duthie et al. 2018). For example, researchers studied the effect of potential factors including sex, age and season (Marlowe and Gaines 1958, Tanner et al. 1970) on the growth of animals in their early life stages, from birth to 120 days old (Windeyer et al. 2014), the first 23 weeks (Lensink et al. 2000) and from birth to weaning age (Soberon et al. 2012). Higher sensitivity of the exponential model in analysing the effects of "intervention" factors had been validated by using the reduced data sets, which included the data thinned to every two days, every four days, weekly, every two weeks, and every four weeks, showing significant effects of intervention by the exponential model while the linear model showed nonsignificant effects of intervention. The R^2 estimates of the exponential model were always higher than those of the linear model. Thus the exponential model was proposed to be the most suitable growth model for cattle in the early life stages, and was recommended to be used to analyse effects of interventions on growth performance – providing better precision and higher statistical power. The R^2 estimates generated by both models did not decrease as the data became sparser, which suggested that both models could work on sparse data of the early life stages. Data simulation could be used to test the statistical power of the exponential model for further investigation.

The growing stage of cattle is also an important life stage, but since animals are often on pasture during this stage, the automatically weighed bodyweights were not available. For beef cattle that were mature or approaching maturity and in finishing systems on farm, the linear model and the negative exponential model both performed well, the negative exponential model being slightly better for animals that were on feed for longer. While this would be expected as a general property of curves – that the shorter a segment is taken the easier it is to fit a straight line – it is also what would be expected from the general class of curves generated by the rate-state differential equations such as the von Bertalanffy – the rapid growth phase that follows the initial exponential curve approximates a linear function, but over time tends to the negative exponential. The present study suggests only that either linear or negative exponential curves would be appropriate, and that the choice might depend on the length of time on feed.

2.5 Conclusion

A workflow for determining the best model for growth of the cattle in different life stages has been developed. The von Bertalanffy model was the best model for whole lifespan growth of Holstein-Friesian cows. By using near-continuous BWs, the exponential model was found to be the most suitable model for cattle in their early life stages up to 125 d, and this model provided more precise parameter estimates, enabled more efficient determination of explanatory variables and improved statistical power compared to the traditional linear model. Both linear model and negative exponential model produced a good fit to growth of the finishing beef cattle. The longer the finishing beef cattle stayed on farm, the more likely it was that the negative exponential model was the best model. The main implications of the study are that an exponential model should be used in preference to a linear model for assessing growth performance in calves in the first three to four months of life, but that in finishing cattle there is no consistent advantage.

2.6 Author contribution

Except that the data for the cows and calves (owned by SRUC) were exported by Ainsley Bagnall of SRUC and the data for the finishing beef cattle (owned by Harbro Ltd.) were firstly cleaned by Paul Johnson of the University of Glasgow, I was responsible for all the data collation, filtering, analyses, visualisation and thesis writing.

Chapter 3 Effects of Ammonia-treated Cereal Grains on Growth Performance of Beef Cattle

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Highlights:

Treatment of maize with ammonia resulted in improved feed-conversion in cattle.

Ammonia treatment increased faecal and ruminal pH.

Ammonia treatment reduced faecal starch concentration.

Ammonia treatment of cereals increases efficiency of cereal utilisation.

Author contributions:

Huang: data curation, formal analysis, visualisation, writing original draft; Jones: data curation, formal analysis, investigation, methodology, writing - review and editing; Compiani & Grossi: investigation, data curation, methodology, formal analysis, project administration, resources, writing - review and editing; Johnson: supervision, formal analysis, visualisation, validation, writing - review and editing; Eckersall: supervision, writing - review and editing; Rossi: conceptualisation, investigation, data curation, methodology, project administration, resources, writing - review and editing; Jonsson: conceptualisation, supervision, investigation, data curation, methodology, project administration, resources, writing, review and editing. All authors, led by Huang.

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Declaration of competing interest:

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3.1 Introduction

Feed quality for livestock can be adversely affected by pests and microorganisms during storage and effective feed preservation is beneficial to animal health. Many approaches have been taken to create an environment that is not conducive to the survival and reproduction of microorganisms and pests: controlling moisture and temperature, creating an anaerobic environment by adding deoxidiser or filling containers with carbon dioxide (CO_2) or nitrogen (N_2) (Raeker 1990, Hashem et al. 2012, Navarro 2012). Additives such as microbial inoculants, chemicals and enzymes, are also commonly used to both preserve and enhance the digestibility and nutritive value of rations for livestock (Muck et al. 2018). Cereal grains contribute a large proportion of the diets for fattening cattle in Europe, and are often harvested with a high moisture content in northern and western Europe, necessitating preservation (Olsson et al. 2002). High starch content of the cereals also increases the risk of reticuloruminal acidosis (Owens et al. 1998). Whereas acidifying treatments are effective preservatives (Campling 1991) but do not address acidosis, alkalization treatments of cereal grains have the potential to address both spoilage and acidosis (Humer and Zebeli 2017).

Ammonia treatment has been applied to inhibit mould growth and bacterial proliferation (Bothast et al. 1972, Kabak et al. 2006) in low quality roughages and whole grains and to increase their digestibility (Oji et al. 1977, Han et al. 1978, Horton 1978, Laksesvela 1981, Kraiem et al. 1991). Ammonia treatment increases non-protein nitrogen (NPN) (Oji et al. 1977, Horton 1978, Herrera-Saldana et al. 1982, Males and Gaskins 1982, Kraiem et al. 1991), which contributes to improved reticuloruminal microbial growth and activity (Rode et al. 1986), and might therefore improve animal performance (Spanghero et al. 2017, Belanche et al. 2021). Improvements in feed intake and feed efficiency in steers (Mathison et al. 1989) and increases in milk yield and milk protein in dairy cows were noted when ammonia-treated high-moisture barley was fed (Robinson and Kennelly 1989). Laksesvela (1981) reported an increase in dry matter intake (DMI) of adult female sheep fed ammonia-treated barley in addition to a higher lambing percentage than those fed untreated barley. Due to the alkalinising nature of ammonia, it is also expected to decrease the rate of ruminal starch degradation like other alkali treatments (Humer and Zebeli 2017), thereby reducing the risk of rumen fermentation disorders.

Ammonia treatment of livestock feed is not new. Early investigations of ammonia made use of anhydrous ammonia gas and demonstrated effective preservation and some enhanced animal performance. However, until recently, relatively little use has been made of ammonia treatment, probably because of the logistic challenges associated with treating large volumes of feed. Several systems have recently been developed commercially, including Maxammon (Harbro Ltd, Turriff, Scotland) in which ammoniation is achieved by mixing cereal grain with urea and a source of enzyme to catalyse the conversion of urea to ammonia. Feed can be treated readily on-farm using mixer wagons, deposited in a commodity-bay, and covered with a plastic sheeting for 7-10 days, during which ammonia gas percolates through the cereal grains and is absorbed. There are fewer concerns about using ammonia as a feed preservative than the ammonia emission in agriculture (from animal husbandry, farming, etc). The amount of ammonia liberated into the atmosphere during the production of the gas is unknown, although in-house studies commissioned by Harbro suggest that it is quite low if the recommendations for sheeting the product are followed. This is to be expected, given the apparent high efficiency with which the ammonia is incorporated in the grains. Maxammon treatment had been reported to improve microbial protein synthesis and therefore improved efficiency of digestion and utilisation of carbohydrates and proteins (Belanche et al. 2021). In the present study, we aimed to establish whether the commercially available method of cereal grain preservation would deliver similar performance benefits to those previously documented using direct insufflation with anhydrous ammonia gas. The beef cattle in the fattening systems were fed on a barley-based diet with ammonia or propionate treatment in a Scotland farm, or were fed on a maize-based diet with or without ammonia treatment in a typical Italian production system. The primary aim was to characterise the performance effects of the enzyme-catalysed ammonia treatment of grain in beef cattle. We hypothesized that cattle fed the ammonia-treated diets would have higher average daily gain (ADG) and lower feed conversion ratio (FCR) than cattle fed the untreated diet or the propionate-treated diet. Secondary aims of the study were intended to suggest possible explanations for any observed differences in performance of animals on the diets and made use of opportunistically collected samples. They included the characterisation of ruminal volatile fatty acid (VFA) profiles and analysis of faeces.

3.2 Materials and methods

Trial 1 was a pilot study, and was carried out on a beef breeding and finishing unit in Aberdeenshire, northeast Scotland, without the use of any regulated procedures under the Animals in Scientific Procedures Act (1986). All data in this trial were collected during the general animal husbandry management of these animals, farmers made the decision on the choice of feed, so no ethical approval was required. Trial 2 was conducted on a commercial beef fattening unit near Milan, in northern Italy, in accordance with the European Communities Council Directive (2010/63/EU), transposed by the Italian Ministry of Health (DL 26, 4 March 2014).

3.2.1 Trial 1 effects of ammonia-treated barley

3.2.1.1 Animals and treatments

Two hundred and seventeen continental crossbreed steers (predominantly Limousin and Charolais; 506 ± 82 days old, 481± 38 kg) were housed in a beef finishing unit near Huntly in Aberdeenshire on 27/07/2017. All animals were treated on arrival on farm against parasites using anthelmintic products (ivermectin and nitroxynil) and were vaccinated against infectious bovine rhinotracheitis (IBR). The animals were allocated to 4 pens (2 pens/treatment group) after stratification on age and weight. There were 62 animals in each of three pens and 31 animals in one pen. Each of the two groups of animals received one of two different diets for 114 ± 10 days, both diets being predominantly barley, but in one diet the barley had been treated with a propionate preservative (PTB) (Prograin, Harbro Limited, Turriff, Scotland) and in the other the barley was treated with an ammonia-producing preservative (ATB) (Maxammon, Harbro Limited, Turriff, Scotland).

All animals were fed three transition diets for 12 to 18 d after being allocated to their groups and before being fed the differently treated diets (Table 3-1). In total, 93 steers were fed ATB, and 124 steers were fed PTB. The barley in both ATB and PTB were prepared by Harbro Limited (Turriff, Scotland). Ammonia treatment of barley was achieved by adding 15 kg of urea and 5 kg of Maxammon (Harbro Limited, Turriff, Scotland) per ton of grain. The moisture content of barley in the study ranged from 17.8 % to 21.0 %, and the inclusion rate of Prograin (Harbro Limited, Turriff, Scotland) varied from 6.5 to 7.5 L/t of grain. Total-mixed rations and all dietary components were analysed by NIR (FossNIRSystems 5000+). The two diets were formulated to be approximately

isoenergetic and isonitrogenous, and to be characteristic of the typical rations fed to finishing cattle in Scotland. The steers were given *ad libitum* access to water and feed.

Table 3-1. Ingredients and average nutritional values of the diets in Trial 1.

	Propionate-treated Barley (PTB)				Ammonia-treated Barley (ATB)			
	1 - 5 d	**6 - 10 d**	**11 - 18 d**	**Final**	**1 - 5 d**	**6 - 8 d**	**9 - 12 d**	**Final**
Ingredient (kg/head/d)								
Pot-ale syrup	3.25	2.89	2.53	3.45	1.75	0	0	0
Molasses	0	0	0	0	0	0.9	0.87	0.96
Straw	1.81	1.08	0.72	0.50	1.83	2.19	0.86	0.58
Prograin Barley	5.06	6.14	7.59	11.33	0	0	0	0
Maxammon Barley	0	0	0	0	6.37	7.7	9.58	13.14
Grampian Beefmax Minerals + Rumitech +Yea-Sacc	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Total as fed	10.25	10.24	10.97	15.41	10.08	10.92	11.44	14.81
DMI	7.1	7.2	7.9	11.1	7.8	9.0	9.4	12.2
Nutrient (% of DM)								
DM	69.7	70.4	71.9	71.9	77.2	82.7	82.4	82.4
CP	13.2	13.3	13	13.3	13.4	10.9	12.3	12.8
NDF	30.4	25.8	23.7	21.6	29.1	30.1	22.1	16.3
Oil	2	2.2	2.4	2.5	2.3	2.4	2.6	1.7
Starch	23.3	27.9	31.4	33.4	33.5	34.8	41.5	43.1
Sugar	2.8	3	3.1	3.2	1.7	3.9	3.8	3.5
Na	0.28	0.26	0.24	0.18	0.26	0.24	0.22	0.18
K	1.04	0.92	0.81	0.77	0.89	0.89	0.76	0.71
Ca	0.63	0.60	0.53	0.41	0.57	0.54	0.48	0.39
Mg	0.21	0.21	0.19	0.19	0.15	0.12	0.13	0.12
Cl	0.52	0.49	0.43	0.33	0.50	0.54	0.48	0.40
P	0.66	0.65	0.61	0.60	0.45	0.27	0.30	0.31
S	0.28	0.26	0.24	0.23	0.22	0.19	0.18	0.18

Maxammon (Harbro Limited, Turriff, Scotland): 15 kg urea and 5 kg Maxammon per ton of barley; Prograin (Harbro Limited, Turriff, Scotland): inclusion ranged from 6.5 to 7.5 L/t of barley; DM: dry matter; CP: crude protein; NDF: neutral detergent fibre.

3.2.1.2 Growth performance

All animals were weighed on D-1, D-58 and D-102 of the trial. The total amount of feed offered to each group of the animals during the trial was recorded every two days. The ADG for each animal was calculated by dividing the difference between the last available weight and the weight on D-1 by the number of days on feed. The FCR of each pen was calculated by dividing the average daily feed intake of each group by the ADG of animals in that group.

3.2.1.3 Ruminal fermentation observations

At slaughter, 50 mL of ruminal fluid from 10 convenience-sampled animals from the ATB group and 9 animals from the PTB group was collected to measure the concentrations of VFAs. Samples were shipped to the laboratory on dry ice, and individual VFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid and heptanoic acid) were measured using gas chromatography (Richardson et al. 1989).

3.2.1.4 Faecal consistency and starch concentration

On 6 occasions, approximately every 2 weeks during the study period, 10 fresh and untrampled faecal samples were collected from the three big pens and 5 - 7 samples were collected from the small pen, taking pains to ensure that the samples were representative of the pen and unlikely to include more than one sample from any single animal. Faeces was scored according to consistency, on a scale of 1 to 5, with 1 being very dry and forming a pile of more than 50 mm high, and 5 being moist to liquid with blood or mucus (Table 3-2), with a high score indicating diarrhoea. In total, there were 95 faecal samples from the ATB group and 120 from the PTB group (30 to 50 mL of faeces per sample). Samples were refrigerated immediately after collection, transported directly to the laboratory, and stored at - 80˚C until use.

Five fresh faecal samples from each pen were collected on D-29 and D-85 and were sent to Scotland's Rural College (SRUC) to measure the starch concentration. Starch was determined using the polarimetric method as described in the European Union Commission Directive for feed (1999/79/EC).

Table 3-2. Scoring system of faeces in Trial 1.

Score	Description
1	Quite dry; excessive fibre evident throughout stool; form piles more than 50 mm high.
2	Moist to wet; porridge-like consistency; clearly defined shape and contour; well-digested fibre is evident.
3	Wet; runny puddle with no contour; little evidence of fibre.
4	Liquid with foam; undigested cereals prominent.
5	Moist to liquid; with or without foam; undigested cereals prominent; fibrin casts, mucus or blood.

3.2.2 Trial 2 effects of ammonia-treated maize

3.2.2.1 Animals and treatments

One hundred and three Charolais cattle (475 ± 39 days old, 454 ± 37 kg) were used in the trial. All animals were treated on arrival against endo- and ectoparasites using a broad-spectrum endectocide and were vaccinated against bovine herpesvirus-1, parainfluenza-3, bovine respiratory syncytial virus and bovine viral diarrhoea virus. After ranking by weight, animals were randomly allocated by initial coin toss and subsequent alternation to 16 pens (5 - 7 animals/pen, 8 pens/group) in two sheds on 19/03/2018. All animals in each pen were fed on the same treated diet for 176 d.

Table 3-3 shows the composition of the two diets, which were intended to be approximately isoenergetic and isonitrogenous, and the average energy and nutrient composition during the experimental period. All components were analysed by near infrared spectrophotometry (NIR) performed by Harbro Limited (Turriff, Scotland) using a FossNIRSystems 5000+ machine, while TMRs were analyzed weekly, through a portable NIR instrument (Polispec, IT Photonics, Italy). In total, 51 cattle were fed the maize-based diet with ammonia treatment (ATM) and 52 cattle were fed the maize-based diet without ammonia treatment (UTM). Maize silage and straw were produced on farm, and other ingredients included in the diet were sourced locally. Ground maize was treated with Maxammon according to the instructions provided by Harbro Limited: 15 kg urea and 5 kg Maxammon per ton of grain, with the addition of 30 L water/t if the moisture content was 14-15 % and an additional 10 L water/t for each additional percentage point below 14 %.

The cattle had *ad libitum* access to hay for 7 d after arrival with progressively increasing portion of TMR (from 50 % to 100 % from D-2 to D-7) . After the initial transition period, cattle had *ad libitum* access to TMR provided every morning at 07:00 h. Cattle always had free access to fresh water.

Table 3-3. Ingredients and average nutritional values of the diets during the experimental period of Trial 2.

	Untreated Maize (UTM)	**Ammonia-treated Maize (ATM)**
Ingredient as fed (kg/head/d)		
Maize Silage	9.0	9.0
Maize meal	5.2	0
Maxammon maize meal	0	6.5
Brewers' grains	3.0	3.0
Straw	1.0	1.0
Rape cake	2.0	1.0
Minerals and vitamins mix	0.2	0.2
Total as fed (kg/head/d)	20.40	20.70
DM as fed (kg/head/d)	11.00	11.00
ME (MJ/kg)	12.05	12.05
Nutrient (% of DM)		
CP	13.78	13.79
Fat	4.11	4.06
NDF	31.37	29.73
Starch	42.23	47.96
Ca	0.70	0.69
P	0.40	0.37

Maxammon (Harbro Limited, Turriff, Scotland): 15 kg urea and 5 kg Maxammon per ton of maize; ME: metabolizable energy; CP: crude protein; NDF: neutral detergent fibre.

3.2.2.2 Health status and growth performance

Animals were inspected daily for lameness, bloat, and signs of bovine respiratory disease (BRD) and weighed on D-1, D-60, D-116 and D-176 (last day) of the trial. The feed intake of each pen was calculated once a week by weighing the feed offered and the residue in the

feed-trough 24 h post-feeding and then corrected for the diet dry matter, evaluated each time through a portable NIR instrument. The ADG of each animal was obtained by dividing the difference between the liveweight on D-176 and D-1 of the trial by 175 (days on feed). The FCR of each pen was calculated by dividing the average daily feed dry matter intake of each pen by the ADG of animals in that same pen.

3.2.2.3 Ruminal fermentation observations

Twenty-eight animals (14 animals per group, with at least 1 animal from each pen) were convenience-selected for post-mortem sampling in the abattoir. Within 30-40 minutes after slaughter, ruminal fluid (200 mL per animal) was collected from the dorsal sac of the rumen for the determination of pH, NH_3, total and proportional volatile fatty acids (lactic acid, propionic acid, butyric acid and acetic acid). The pH was measured immediately on collection using a portable pH-meter (HI 5522, HANNA Instruments, Woonsocket, USA). The concentration of NH_3 was measured using a modified colorimetric method developed by Broderick and Kang (1980). The VFA concentrations were analysed using high-performance liquid chromatography (Shimatzu, Kyoto, Japan).

3.2.2.4 Faecal consistency, undigested fraction, and chemical characteristics

Faeces were collected from 28 animals (14 animals were random-sampled per group and avoiding animals under medical treatment) on D-60, D-116 and D-176. Faecal consistency was scored from 1 to 5, 1 being very compact, semi-solid and 5 being very liquid, using a method based on criteria discussed by Hall (2002). Faecal fractional size profile was estimated by sieving faeces with a 3-plate sieve, with sieve dimensions of 4.76 mm, 3.17 mm and 1.55 mm. The pH of the faeces was measured using a portable pH-meter (HI 5522, HANNA Instruments, Woonsocket, USA). Faecal measurements were carried out according to the AOAC guidelines (1990): faecal moisture content (method 934.01); concentrations of crude protein (CP) (method 920.87); lipids (method 920.85) and starch (method 996.11). The concentrations of faecal ADF and NDF were measured using the method described by Van Soest et al. (1991).

3.2.3 Statistical analysis

Data were analysed in R version 4.0.3 (R Core Team, 2020). The effects of treatments and other potential factors (shed, pen or sampling day) were tested by fitting a linear mixed-effects model (LMM) using the *lme4* package (Bates et al. 2015) or a generalized linear model (GLM). The distributional assumptions of LMMs and GLMs were checked visually by plotting residuals against fitted values. Variables such as ruminal NH_3 concentration and faecal starch concentration required natural-log-transformation to achieve normally distributed residuals. Significance was defined at $p < 0.05$ and a trend was defined at $0.05 \leq p < 0.10$.

Trial 1 was a pilot study, and although the number of animals was quite large, there were only two pens for each treatment group. For pen to be considered as a random effect, at least 4 pens would be required per group. Models below and *F*-test in *anova* function in R were used to give some indication as to whether it would be reasonable to infer whether any effect might arise from the pen or from the treatment. If there was no evidence of a significant pen effect ($p > 0.05$), a model with the treatment as a fixed effect might be applied with caution. Although there were only two pens for each treatment group, pen was used as the experimental unit of observations as well, to test the treatment effects on ADG and FCR.

The ADG and FCR were analysed using a GLM:

$$Y_{ik} = \mu + T_i + e_{ik}$$

or

$$Y_{nk} = \mu + P_n + e_{nk}$$

Where Y_{ik} is the dependent, continuous variable; μ is the mean of all observations when animal is the unit or the average value of mean of pen observations when pen is the unit; T_i is the fixed effect of the diet (i = ATB and PTB); P_n is the fixed effect of the pen (n = 1 to 4); e_{ik} and e_{nk} are the normally distributed residual errors.

For the number of cattle that had faecal score of 3, 4 or 5, or faecal score of 4 or 5, binary logistic regression in GLM was applied:

$$L_i = logit^{-1}\ (\mu + T_i),\ Y_{im} \sim Bernoulli\ (L_i)$$

or

$$L_n = logit^{-1}\ (\mu + P_n),\ Y_{nm} \sim Bernoulli\ (L_n)$$

Where L_i or L_n is the probability that Y_{im} or Y_{nm} is assigned to the categories 0 or 1; $\mu + T_i$ or $\mu + P_n$ is the predicted log odds, i = ATB and PTB and n = 1 to 4. The two models were compared using the *LRT* (likelihood ratio test) in *anova* function in R to test the effect of the pen. Where there was no evidence of an effect of pen ($p > 0.05$), the model could be applied (with caution) to analyse the effect of the diet on the faecal score. Pen was also used as the experimental unit of observations, using count of animals of which the faecal score was either ≥ 3 or ≥ 4 in GLMs.

The model for analysing ruminal content was:

$$Y_{ik} = \mu + T_i + e_{ik}$$

Where Y_{ik} is the dependent variable; μ is the overall mean; T_i is the fixed effect of the diet (i = ATB and PTB), e_{ik} is the residual error.

For the faecal starch and DM concentration analysis, a GLM was applied:

$$Y_{imk} = \mu + T_i + D_m + (T \times D)_{im} + e_{imk}$$

Where Y_{imk} is the dependent, continuous variable; μ is the overall mean; T_i is the fixed effect of the diet (i = ATB and PTB); D_m is the fixed effect of the sampling day (m = D-29 and D-85); $(T \times D)_{im}$ is the interaction between the diet and the sampling day and e_{imk} is the normally distributed residual error. Analysis of deviance for the fit of the GLM (F-test

in *anova* function) was performed to compare the model with the interaction term to the model with main effects only - the interaction term was removed if $p > 0.10$.

In Trial 2, the cattle were allocated to two sheds; four pens of cattle in each shed were fed one of two diets (ATM or UTM).

The ADG and FCR were analysed using LMM:

$$Y_{ijnk} = \mu + T_i + S_j + (T \times S)_{ij} + P_n + e_{ijnk}$$

Where Y_{ijnk} is the dependent, continuous variable; μ is the overall mean; T_i is the fixed effect of the diet (i = ATM and UTM); S_j is the fixed effect of the shed (j = 1 and 2); $(T \times S)_{ij}$ is the interaction between the diet and the shed; P_n is the normally distributed random effect of the pen (n = 1 to 16) and e_{ijnk} is the normally distributed residual error. The interaction between the diet and the shed was removed when $p > 0.10$, and the resulting model was fitted again to evaluate the effects.

For the faecal data collected over time, the same GLM as used in Trial 1 was applied, except that T_i is the fixed effect of the diet (i = ATM and UTM); D_m is the fixed effect of the sampling day (m = D-60, D-116 and D-176). GLMs with the interaction term and with main effects only were compared, and the interaction term was removed if $p > 0.10$.

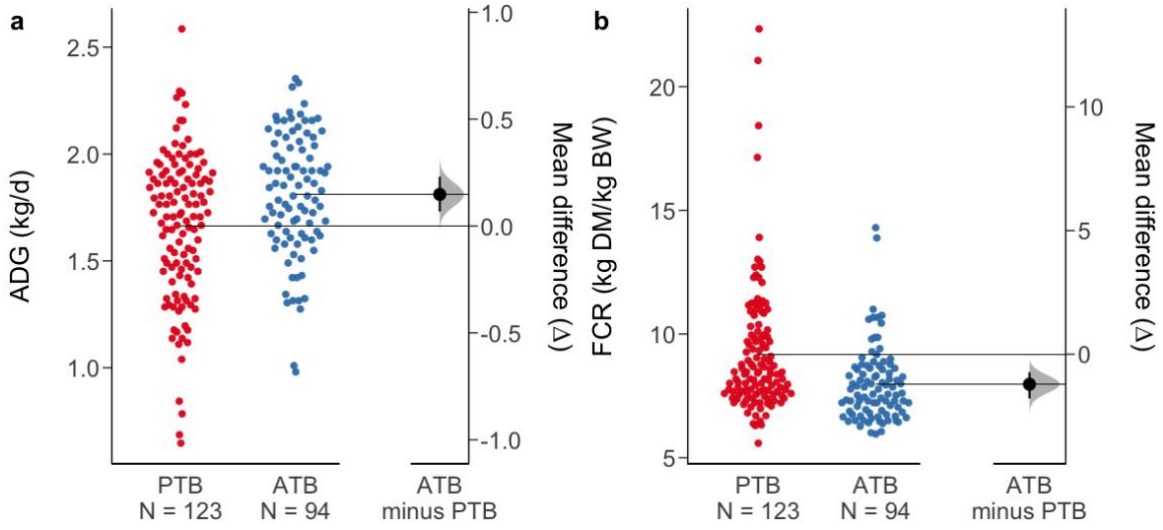
The model for analysing ruminal content was the same as in Trial 1, except that T_i is the fixed effect of the diet (i = ATM and UTM).

3.3 Results

3.3.1 Trial 1 effects of ammonia treatment of barley

3.3.1.1 Animal growth performance

There was no effect of pen in this pilot study. When pen was used as the experimental unit of observations, no significant effects of the treatment on final BWs (p = 0.11) and ADG (p = 0.15) of cattle were found. However, the FCR of cattle that were fed the ATB diet tended to be lower than those fed the PTB diet (p = 0.092). When animal was regarded as the experimental unit, the final BWs of animals in the ATB group tended to be heavier compared to those in the PTB group (664 ± 57 vs 650 ± 57 kg, p = 0.073). Animals fed the ATB diet had higher ADG when compared with those fed the PTB diet (1.81 ± 0.29 vs 1.66 ± 0.34 kg/d; p < 0.001), and the FCR of animals fed the ATB diet was lower than those fed the PTB diet (9.18 ± 2.60 vs 7.97 ± 1.50 kg DM/kg BW; p < 0.001) (Figure 3-1 and Table 3-4).



Gardner-Altman plots show the individual mean values for each animal on the left, the mean difference of the ATB mean from the PTB mean (dark horizontal line), and the distribution of the deviations of the ATB observations from the PTB mean (shaded in grey).

Figure 3-1. (a) The average daily liveweight gain (ADG) and (b) feed conversion ratio (FCR) of cattle fed the diet containing ammonia-treated barley (ATB) and the diet containing propionate-treated barley (PTB).

Table 3-4. Effects of feeding the ammonia-treated barley (ATB) on growth performance and concentrations of ruminal VFAs of beef cattle compared to those fed the propionate-treated barley (PTB).

	PTB	ATB	p - value (animal as unit)	p - value (pen as unit)
Growth performance	n = 114 cattle	n = 93 cattle		
Initial BW (kg)	483 ± 38	480 ± 38	0.50	0.24
Final BW (kg)	650 ± 57	664 ± 57	0.073	0.11
ADG (kg/d)	1.66 ± 0.34	1.81 ± 0.29	< 0.001	0.15
FCR (kg DM/kg BW)	9.18 ± 2.60	7.97 ± 1.50	< 0.001	0.092
Ruminal content	n = 9 cattle	n = 10 cattle		
VFA (mmol/L)	194.74 ± 73.52	187.46 ± 85.04	0.85	-
Acetic acid (% VFA)	52.34 ± 4.43	58.67 ± 3.61	0.0032	-
Propionic acid (% VFA)	25.15 ± 5.81	18.51 ± 1.90	0.0032	-
Butyric acid (% VFA)	9.19 ± 1.16	9.46 ± 2.64	0.78	-
Isovaleric acid (% VFA)	5.53 ± 2.17	6.85 ± 2.15	0.20	-
Isobutyric acid (% VFA)	3.42 ± 1.10	3.67 ± 1.02	0.60	-
Valeric acid (% VFA)	3.19 ± 0.76	2.10 ± 0.33	< 0.001	-
Hexanoic acid (% VFA)	0.99 ± 0.39	0.62 ± 0.41	0.067	-
Heptanoic acid (% VFA)	0.19 ± 0.16	0.10 ± 0.19	0.29	-
Acetate: Propionate	2.22 ± 0.69	3.20 ± 0.38	0.0012	-

PTB: diet contains propionate-treated barley; ATB: diet contains ammonia-treated barley; BW: bodyweight; data are presented by mean ± SD; the *p* - values were obtained by fitting the dependent variables with the GLM with the diet as a fixed effect.

3.3.1.2 Ruminal fluid characteristics at slaughter

Ruminal acetic acid, propionic acid and valeric acid as proportions of the total VFAs, and the ratio of acetic acid to propionic acid differed between groups. The proportion of acetic acid in total VFAs ($p = 0.0032$) and the ratio of acetic acid to propionic acid ($p = 0.0012$) were higher in the ATB group when compared to the PTB group, whereas propionic acid ($p = 0.0032$) and valeric acid ($p < 0.001$) as proportions of total VFAs of the cattle fed ATB were lower than those fed PTB. The proportion of hexanoic acid in total VFAs tended to be lower in ATB than that in PTB ($p = 0.067$). No differences were found in the concentration of the total VFA (Table 3-4).

3.3.1.3 Faecal consistency and DM and starch concentrations

No effect of pen was found on faecal scores in the pilot study. In the PTB group, 17.5 % of faecal samples scored 4 or 5 and 36.7 % of faeces were scored 3 or 4 or 5. However, only 3.2 % of faecal samples in the ATB group scored 4 or 5, and only 17.9 % of faeces in the ATB group were scored 3 or 4 or 5. Whether using animal or pen as the experimental unit, significant effects of the treatment on faecal scores were detected (Table 3-5). The sampling day tended to affect faecal DM concentration ($p = 0.082$). The concentration of faecal starch was lower ($p < 0.001$) in the ATB group (24.59 ± 19.36 g/kg DM) than in the PTB group (61.17 ± 47.94 g/kg DM), and there might be an effect of the interaction between the diet and sampling day ($p = 0.083$).

Table 3-5. Effects of the ammonia-treated barley on faecal consistency, dry matter and starch concentrations compared to the propionate-treated barley.

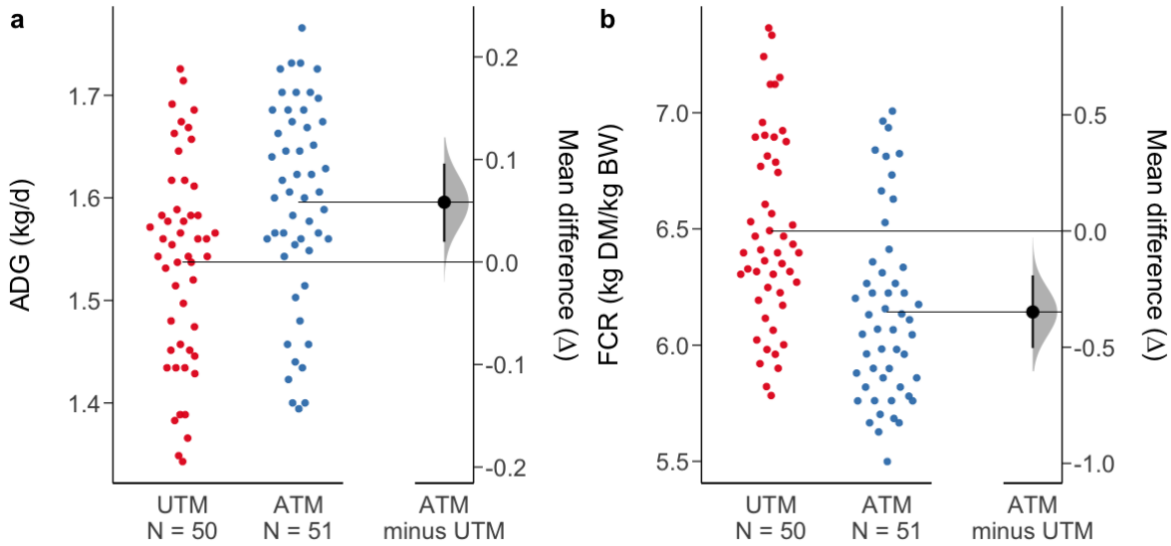
	Diet		**Day**		**p - value**	**p - value**	**p - value**
	PTB	**ATB**	**D-29**	**D-85**	**(diet)**	**(day)**	**(diet × day)**
	n = 20	n = 20	n = 20	n = 20			
DM (%)	14.59 ± 2.76	14.83 ± 2.46	13.99 ± 2.47	15.43 ± 2.54	0.76	0.082	-
Starch (g/kg DM)	61.17 ± 47.94	24.59 ± 19.36	42.56 ± 34.89	43.19 ± 46.48	< 0.001	0.32	0.083
Count	n = 120	n = 95			**p - value (animal as unit)**	**p - value (pen as unit)**	
Faecal score of 4 or 5	21 (17.5 %)	3 (3.2%)			0.0031	0.030	
Faecal score of 3, 4 or 5	44 (36.7 %)	17 (17.9 %)			0.0029	0.0068	

PTB: diet contains propionate-treated barley; ATB: diet contains ammonia-treated barley; continuous variables are presented by mean ± SD; the *p* - values of the effects on DM and starch concentrations were obtained by fitting the GLMs with the diet, the sampling day and their interaction as fixed effects; the effects of the diet on faecal scores were obtained by fitting binary logistic regression in GLM with the diet as a fixed effect when using animal as the experimental unit of observations, or by fitting GLM with the count of observations of each pen with the diet as a fixed effect when using pen as the unit.

3.3.2 Trial 2 effects of ammonia treatment of maize

3.3.2.1 Health and performance

During the study, two cattle that were fed UTM were slaughtered before the expected time on feed had elapsed – one due to severe BRD and one due to severe bloat. Twenty-three of fifty-two cattle in the UTM group and 21/51 cattle in the ATM group were diagnosed with BRD. There were eight lame cattle in the UTM group and six in the ATM group; seven cattle in the UTM group and three in the ATM group had bloat that was responsive to treatment or recovered with no treatment. Animals fed the ATM diet tended to have a higher ADG than those fed the UTM diet (1.60 ± 0.10 vs 1.54 ± 0.10 kg/d; $p = 0.060$). The FCR of animals fed ATM was lower than those fed the UTM diet (6.14 ± 0.39 vs 6.49 ± 0.41 kg DM/kg BW; $p = 0.011$; Figure 3-2).



Gardner-Altman plots show the individual mean values for each animal on the left, the mean difference of the ATM mean from the UTM mean (dark horizontal line), and the distribution of the deviations of the ATM observations from the UTM mean (shaded in grey).

Figure 3-2. (a) The average daily liveweight gain (ADG) and (b) feed conversion ratio (FCR) of cattle fed the diet containing ammonia-treated maize (ATM) and the diet containing untreated maize (UTM).

3.3.2.2 Ruminal fluid characteristics at slaughter

The pH of the ruminal fluid at slaughter was higher ($p < 0.001$) in animals that were fed the ATM diet (6.35 ± 0.69) compared to those fed the UTM diet (5.46 ± 0.33). No differences were found in the concentrations of NH_3 or total VFA in ruminal fluid between the two groups. However, the proportions of butyric acid ($p = 0.012$) and propionic acid ($p < 0.001$) in the total VFAs differed between groups. The proportion of butyric acid was 14.69 ± 3.55 % of the total VFAs in animals fed the ATM diet, while it was 18.04 ± 2.97 % of the total VFAs in the UTM fed group. The proportion of propionic acid was 22.14 ± 1.54 % of the total VFA in the ATM group but only 19.54 ± 1.72 % VFA in the UTM group. The ratio of acetic acid to propionic acid was lower in animals fed ATM compared to those fed UTM (ATM vs UTM: 2.64 ± 0.26 vs 2.97 ± 0.29; $p = 0.0038$). There was no difference in the concentration of lactic acid between groups, nor were there any differences in the proportion of acetic acid within the total VFA concentration (Table 3-6).

Table 3-6. Effects of feeding the ammonia-treated maize on growth performance and ruminal content at slaughter of beef cattle.

	Untreated Maize (UTM)	Ammonia-treated Maize (ATM)	p - value (diet)	p - value (shed)
Growth performance	n = 50 cattle	n = 51 cattle		
Initial BW (kg)	458 ± 38	450 ± 36	0.41	0.02
Final BW (kg)	727± 24	729 ± 25	0.93	0.0089
ADG (kg/d)	1.54 ± 0.10	1.60 ± 0.10	0.060	0.10
Feed efficiency	n = 8 pens	n = 8 pens		
DMI (kg/d)	10.18 ± 0.48	9.82 ± 0.42	0.18	0.22
FCR (kg DM/kg BW)	6.49 ± 0.41	6.14 ± 0.39	0.011	0.19
Ruminal content	n = 14 cattle	n = 14 cattle	*p* - value (diet)	
pH	5.46 ± 0.33	6.35 ± 0.69	< 0.001	
NH3 (mmol/L)	19.22 ± 8.82	21.28 ± 10.84	0.88	
VFA (mmol/L)	167.84 ± 26.77	154.32 ± 31.70	0.23	
Lactic acid ((% VFA)	1.39 ± 1.19	0.78 ± 0.44	0.19	
Acetic acid (% VFA)	57.67 ± 2.12	58.20 ± 2.53	0.55	
Butyric acid (% VFA)	18.04 ± 2.97	14.69 ± 3.55	0.012	
Propionic acid (% VFA)	19.54 ± 1.72	22.14 ± 1.54	< 0.001	
Acetic acid: Propionic acid	2.97 ± 0.29	2.64 ± 0.26	0.0038	

Two animals in the UTM group were removed from the analysis because of the death; continuous variables are presented by mean ± SD; the *p* - values of effects on growth performance were obtained by fitting the variable with the LMM with the diet and the shed as fixed effects, the pen as random effects; the effects on ruminal content were analysed using the model with the diet as a fixed variable.

3.3.2.3 Faecal characteristics

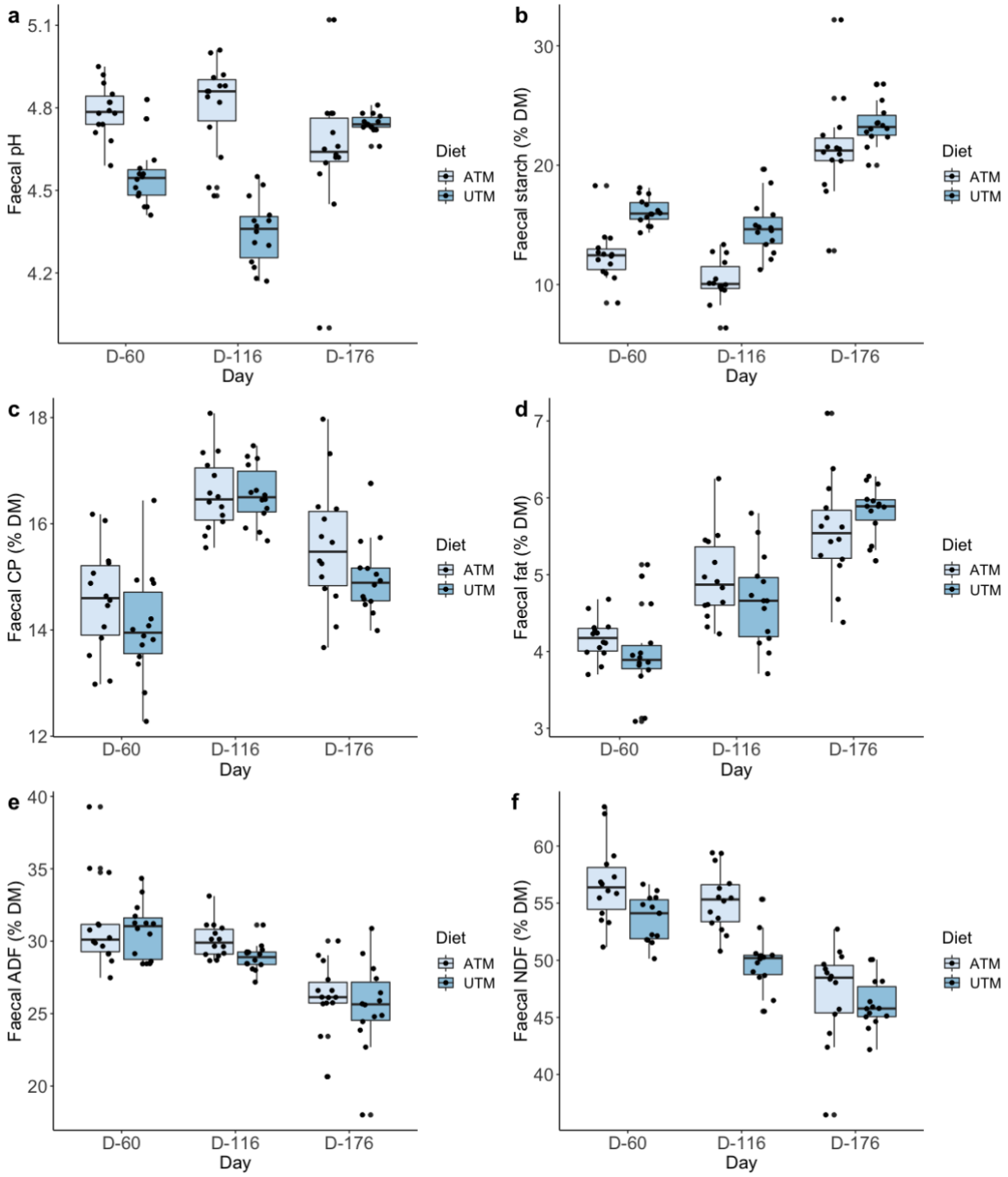
No differences were found in the faecal consistency between groups. The UTM and ATM groups (42 faecal samples per group) had 28 and 27 faecal samples respectively that were scored 4 or 5. The remaining samples in both groups were scored 3. No differences were found in the results of 3-level sieving between groups (Table 3-7). However, faecal composition differed between the two groups and differed over time (Table 3-7, Figure 3-3). The pH of faeces in the ATM group was higher than that in the UTM group (4.75 ± 0.19 vs 4.55 ± 0.19; $p < 0.001$). The faecal starch concentration in the ATM group was lower than that in the UTM group (14.73 ± 5.65 vs 18.14 ± 4.27 % DM; $p < 0.001$). The proportion of NDF in faecal DM of the cattle in the ATM group was higher than that in the UTM group (53.04 ± 5.45 vs 49.91 ± 3.77 % DM; $p < 0.001$). All the faecal composition

variables changed significantly over time ($p < 0.001$) and interactions between the diet and sampling day affected pH ($p < 0.001$), NDF proportion ($p = 0.022$) and starch concentration ($p = 0.0097$) in faeces. The proportion of CP in faecal DM tended to be higher in the ATM group when compared to the UTM group (15.57 ± 1.27 vs 15.20 ± 1.29 % DM; $p = 0.059$). No significant differences in faecal DM proportion, or proportions of fat and ADF in faecal DM caused by the ammonia treatment were found.

Table 3-7. Effects of feeding the ammonia-treated maize on faecal consistency and composition of beef cattle.

	Diet		Day			p - value (diet)	p - value (day)	p - value (diet × day)
	UTM (n = 42 cattle)	ATM (n = 42 cattle)	D-60 (n = 28 cattle)	D-116 (n = 28 cattle)	D-176 (n = 28 cattle)			
Physical evaluation								
Sieve I (%)	3.07 ± 1.22	3.00 ± 1.43	3.07 ± 1.33	3.14 ± 1.53	2.89 ± 1.10	0.81	0.77	-
Sieve II (%)	16.43 ± 3.90	16.62 ± 3.13	16.61 ± 4.24	16.68 ± 2.89	16.29 ± 3.40	0.81	0.91	-
Sieve III (%)	80.50 ± 4.30	80.38 ± 3.58	80.32 ± 4.38	80.18 ± 3.78	80.82 ± 3.72	0.92	0.81	-
Chemical evaluation								
pH	4.55 ± 0.19	4.75 ± 0.19	4.67 ± 0.16	4.58 ± 0.27	4.69 ± 0.18	< 0.001	0.0094	< 0.001
DM (%)	19.96 ± 1.44	20.14 ± 1.38	21.24 ± 1.20	19.47 ± 1.22	19.32 ± 0.97	0.48	< 0.001	-
CP (% of DM)	15.20 ± 1.29	15.57 ± 1.27	14.32 ± 1.02	16.56 ± 0.63	15.28 ± 1.00	0.059	< 0.001	-
Fat (% of DM)	4.83 ± 0.92	4.90 ± 0.78	4.08 ± 0.46	4.81 ± 0.59	5.70 ± 0.55	0.55	< 0.001	-
NDF (% of DM)	49.91 ± 3.77	53.04 ± 5.45	55.19 ± 3.18	52.57 ± 3.70	46.67 ± 3.32	< 0.001	< 0.001	0.022
ADF (% of DM)	28.38 ± 3.01	29.17 ± 3.13	30.94 ± 2.56	29.47 ± 1.23	25.92 ± 2.69	0.11	< 0.001	-
Starch (% of DM)	18.14 ± 4.27	14.73 ± 5.65	14.30 ± 2.56	12.56 ± 3.05	22.45 ± 3.40	< 0.001	< 0.001	0.0097

UTM: untreated maize; ATM: ammonia-treated maize; continuous variables are presented by mean ± SD; dimensions of sieve I to III are 4.76 mm, 3.17 mm and 1.55 mm, respectively; the *p* - values were obtained by fitting the variable with the GLM with the diet, the sampling day and their interaction as fixed effects.



Sampling day significantly affected all the variables ($p = 0.0094$ for pH and $p < 0.001$ for all the other variables), and the main effect of treatment was significant for pH, NDF and starch ($p < 0.001$) and CP tended to be higher in ATM animals ($p = 0.059$). Significant interactions of sampling day by treatment were seen for pH, NDF and starch ($p < 0.001$, $p = 0.022$ and $p = 0.0097$, respectively).

Figure 3-3. The effect of feeding the ammonia-treated maize (ATM) on (a) faecal pH, and faecal proportions of (b) starch, (c) CP, (d) fat, (e) ADF, (f) and NDF.

3.4 Discussion

In the present study, the diets were broadly equivalent in energy and protein. The study was intended to contrast the performance of cattle fed on an ammonia-treated cereal diet in comparison with a locally more traditional diet, in two distinct commercial settings. In Scotland, where preservation of cereal grains is essential, the comparison was between propionate treatment with Prograin (Harbro, Scotland) and ammonia treatment with Maxammon (Harbro, Scotland). In northern Italy, where preservation was not required, the comparison was between Maxammon and a control system with no preservative. The whole study was intended to contrast the effect of the feeding system rather than any independent, direct effect of the ammonia treatment for two main reasons. Firstly, the study was conducted on commercial farming units to maximise the relevance of the results to cattle producers. This imposes considerable constraints on the types of rations to be fed and an expectation that each of the alternative treatments be used in a way that maximises the potential productivity of animals given each treatment. Nested within this reason, the ammonia treatment has been adopted for use by farmers partly on the basis that its presumed buffering effect should enable higher rates of starch to be fed than when using other preservatives (anecdotal information provided to Nicholas Jonsson and Rheinallt Jones by Scottish farmers and feed suppliers). Secondly, the ammonia treatment provides an additional source of NPN, which must be balanced somehow, using a comparison that is commercially plausible. A treatment with urea in the absence of enzymes to catalyse its conversion to ammonia would not be feasible in this study because some (possibly most) of the urea would be expected to be converted to ammonia as a result of endogenous cereal enzymes (Patra and Aschenbach 2018), reducing the validity of the control treatment.

The rates of BRD in the Italian study are broadly consistent with expectation, since the prevalence of BRD in Italy can be up to 80 % (Timsit et al. 2016, Padalino et al. 2021). Any disease in a study like this tends to have a diluting effect on the treatments under investigation, that is, there is a tendency to underestimation. There were BRD cases in the pilot study as well, and the incidence of BRD in the pilot study could have been higher because there was not a protocol for veterinary examination and reporting was by the farmer. Sick animals were not removed from analysis, making the data in the figures like outliers. An alternative method of analysis is by proportions of animals in classes of performance.

An ideal study would provide individual treatments to group-housed animals such that there is no effect of pen or group and the individual animal would be truly the experimental unit. However, this can only be achieved with automated, multi-channel, individual feeding units. In the pilot study, there were only two pens in each treatment group, so that pen was not considered as a random effect in fitting GLMs. No evidence of significant pen effect was found, therefore the analyses in this trial used the treatment as a fixed effect. Although only two pens per group is not ideal, there were large number of observations in each pen. Using pen as the unit is a conservative approach and has been applied in many studies, thus it was used as the unit in some analyses in this trial when available, to test the effect of the treatment. No significant effect of the treatment on ADG was found when using pen as the experimental unit, but the FCR of animals that were fed ATB tended to be lower than those fed the PTB diet ($p = 0.092$). As expected, the apparent effects of the treated diet on ADG and FCR were higher when using animal as the experimental unit. It indicated that the ADG of cattle in ATB was higher and the FCR which were fed ATB was lower than those fed PTB ($p < 0.001$). Although the analyses with pen as the experimental unit is weak, and the legitimacy of using individual animal as the unit of study might be debated, the approximate size and direction was considered sufficient to justify progressing with the Italian study. Consistent with the pilot study and with earlier studies in which ammoniation was achieved through direct gaseous insufflation (Phillip et al. 1985, Goonewardene et al. 1998), in the Italian full study the FCR of cattle which were fed the ATM diet was lower than those fed the UTM diet. The ADG of cattle which were fed the ATM diet tended to be higher than those fed the UTM diet. Hence, there was an observed FCR benefit of 5.4% (0.35/6.49 kg/kg, $p = 0.011$) – broadly consistent with the DMI being 3.7% less in the animals on the ATM diet (0.36/10.18 kg/d, $p = 0.18$) and the ADG being 3.8% higher (0.06/1.60 kg/d, $p = 0.06$). A change for a p value between 0.05 and 0.1 was considered as a tendency, since small gains in efficiency can have large effects on profitability. For example, it has been reported that an average net margin per head for finishing units in Scotland was about £142 over a total revenue of £1520 (2022), which equates to less than 10%, same in some other places or even lower. It is possible that the performance benefits seen with ammonia treatment arise from improved digestibility of the cereal grains, as well as increased nutrient utilisation attributed to the increased microbial growth arising from nitrogen. The improved digestibility of whole DM (Ørskov et al. 1982), organic matter (OM) (Laksesvela 1981) or crude fibre (Low and Kellaway 1983, Mandell et al. 1988) of whole moist barley due to the ammonia treatment have been reported, while Robinson and Kennelly (1989) and a recent study which used Maxammon (Belanche et al. 2021) did not detect an effect on digestibility of OM. However, higher *in*

sacco N degradability and a tendency to higher total tract apparent N digestibility of barley were noted by Belanche et al. (2021), and Low and Kellaway (1983) reported a significant increase in apparent N digestibility of whole wheat grain due to the ammonia treatment. Ammonia treatment has been shown to increase the production of urinary purine derivatives and microbial DNA, suggesting higher microbial protein synthesis (Belanche et al. 2021). It might be expected that this effect would be similar if feed was ammonia-treated or if it was simply supplemented with urea, because urea in the rumen is converted to ammonia, which is the key nitrogen substrate for microbial growth (Patra and Aschenbach 2018). However, cereal that has been insufflated with ammonia gas (either directly or by pre-feeding treatment with urea and enzyme), the ammonia has been incorporated within the cereal grain (Nikulina et al. 2018). In *in vitro* studies, ammonia in ruminal fluid builds more slowly after ingestion of ammonia-treated cereal than cereal to which an equivalent amount of urea had been added (Nikulina et al. 2018). It is possible that this slower release of ammonia might provide more stable conditions in the rumen for microbial protein synthesis, as suggested by higher urinary purine derivatives and microbial DNA in sheep on ammonia-treated diets (Belanche et al. 2021).

Higher ruminal microbial activity would be expected to result in more rapid and complete utilisation of available starch within the rumen. In both the Scottish pilot study and the Italian full study, the faecal starch concentrations of cattle which were fed ammonia-treated grains (ATB and ATM) were lower than those fed the other diets (UTM and PTB), which was consistent with either increased digestibility of the grain or increased utilisation (Laksesvela 1981, Zinn et al. 2007, Fredin et al. 2014). The inferior performance of the propionate treatment in starch utilisation compared to the ammonia treatment in the pilot study might have resulted from the low ruminal pH, which could affect the ruminal microbial composition and activity (Russell and Wilson 1996, Petri et al. 2012). Alkalization of cereal grain has been proposed to reduce the risk of rumen fermentation disorders (Ørskov 1979, Robinson and Kennelly 1988, 1989, Huntington et al. 2006, Humer and Zebeli 2017). The observations from our studies were broadly consistent with this: fewer animals fed on the ammonia-treated maize developed bloat, although bloat incidence was low overall and not tested statistically (8 cases in total fed on UTM *versus* 3 cases on ATM); fewer animals fed on the ammonia-treated barley had higher faecal scores, which were considered to be indicative of diarrhoea, than those fed on the propionate-treated barley. Runny faeces are bad for finishing cattle businesses. From an animal health point of view, they are sick, and there will be a requirement for more straw and more frequent replacement of bedding to reduce problems with feet and legs if there are runnier

faeces. The carcases should be clean or have been cleaned beforehand of slaughter. However, animals' carcases are more contaminated with runnier faeces, meaning that they must sometimes be clipped for slaughter or processed more slowly. Faecal and ruminal pH were also higher in animals on the ATM diet, which was consistent with the findings of Belanche et al. (2021) in sheep.

Volatile fatty acids are often used for evaluation of the effect of differing diets and dietary treatments on ruminal fermentation (Hall et al. 2015). There were no differences in ruminal total VFA concentrations between groups in both the Scottish pilot study and the Italian full study, which were consistent with a study of sheep (Belanche et al. 2021). A higher proportion of ruminal propionic acid and lower butyrate proportion were noted in cattle that were fed on the ATM diet than those fed on the UTM diet. Consistent with this result, Laksesvela (1981) reported increased propionic acid in the ruminal fluid of cattle that were fed ammonia-treated barley. A higher proportion of ruminal acetic acid in cattle that were fed on the ATB diet than those fed on the PTB diet might suggest more active cellulose and hemicellulose degradation in the rumen, which was also reported in (Belanche et al. 2021). Inconsistency among studies in the results of VFA analysis, such as higher proportion of proionate acid and proportion of valeric acid and lower ratio of acetate to propionate in the PTB group compared to the ATB group in the pilot study, and lower ratio of acetate to propionate in the ATM group compared to the UTM group in the Italian full study, may arise in part from the timing of sampling in relation to the dietary change and to the time of the last meal. Cattle on a higher rate of starch supplementation are likely to adapt by developing a higher rate of VFA clearance, which in turn would result in lower concentrations of VFAs if sampling was carried out post-mortem and several hours after the last substantial meal (Jonsson et al. 2019).

Changes in the composition of single faecal samples in cattle should be interpreted cautiously due to substantial diurnal variation in their relative compositions (Jancewicz et al. 2016). Although the effect of the treatment on faecal starch was significant in the pilot study, the interaction between the sampling day and the treatment was not. In the Italian full study, faecal variation over time-points was pronounced for all chemical variables but not for physical or structural variables. The most notable effects were the treatment × sampling day interactions for pH and proportions of starch and NDF where the steers were fed on the ATM diet or UTM diet. Faecal pH was higher in animals on ATM at the first two time points (D-60 and D-116) but by the third time point (D-176) they were similar in both groups, likely due to progressive adaptation of all animals to the high starch diet. In

both trials, lower faecal starch concentrations were measured in the groups fed on ammonia-treated grains. The reason for the increase seen in faecal starch concentration in animals on both diets by the third time-point in the Italian full study requires further investigation. Matthé et al. (2003) demonstrated that faecal starch absorption was maximal at 94.5% when cows were given a 21-d adaptation period. Although the composition of the diet was fixed in our study after the adaptation periods, the absolute feed intake of animals increased as the animals grew, so it was possible that the high faecal starch proportions seen by the end of the feeding period might result from saturation of absorptive capacity in both groups. This might also involve an allometric scaling relationship between body-mass (BM) and absorptive capacity, in which gut fill scales to $BM^{1.0}$ but the rate of metabolic and absorptive processes scales to $BM^{0.75}$ (see (Matthé et al. 2003) for example). Faecal NDF was slightly higher in ATM animals at the first two time points but decreased in both groups over time and were similar by the last time-point. The significant reduction in NDF and ADF in faecal DM over time of the animals that were fed either ammonia-treated maize or untreated maize suggested progressive adaptation to the diets with improved fibre degradation. The effect of NDF and absence of an effect on ADF in faeces of animals in the Italian study were consistent with the variability in results of other studies. Several studies reported no changes in apparent total tract digestibility (aTTD) of NDF and ADF in ruminants resulting from the ammonia treatment of cereal grains (Mathison et al. 1989, Robinson and Kennelly 1989), whereas Mowat et al. (Mowat et al. 1981) and Low and Kellaway (1983) reported improved aTTD of ADF and NDF in steers with ammonia treatment of maize and wheat respectively.

3.5 Conclusion

The enzyme-catalysed ammonia treatment of grain decreased FCR and faecal starch concentrations of cattle, having similar effects of improving the growth performance of cattle in beef fattening systems to those previously reported in studies using direct insufflation with anhydrous ammonia. This method of processing cereal grains has the potential to increase nutrient utilization on commercial cattle farms.

3.6 Author contribution

The experiments of Trial 1 were done by Gui Wolff and Nicholas Jonsson. The experiments of Trial 2 were done by Riccardo Compiani, Carlo Sgoifo Rossi and Silva Grossi of the University of Milan. I was responsible for the data collation, curation, analyses, visualisation and thesis writing.

Chapter 4 In-gel Sample Preparation Prior to Proteomic Analysis of Bovine Faeces Increases Protein Identifications by Removal of High Molecular Weight Glycoproteins

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Declaration of competing interest:

The authors declare no conflict of interests.

Highlights:

The interference of high molecular weight glycoproteins on preparation of faecal samples eliminated.

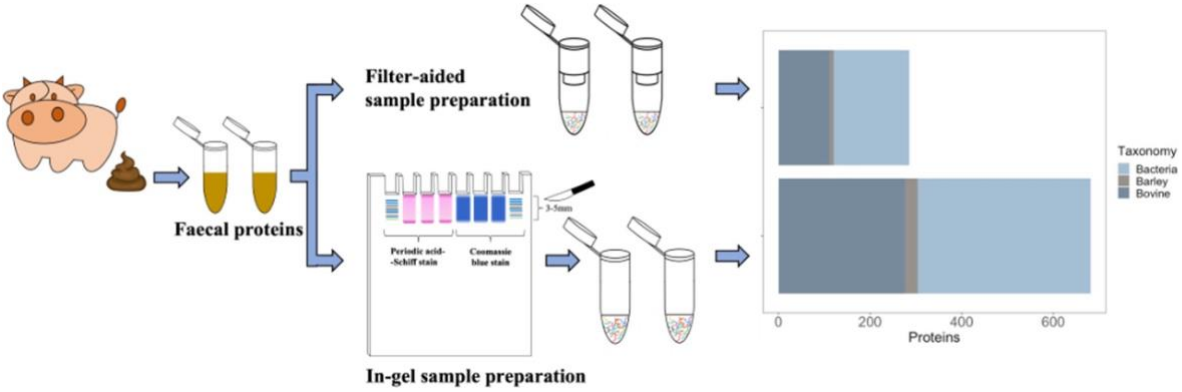
In gel sample preparation increased the number of proteins identified and the number of peptides found per protein.

The bovine faecal proteome is a complex mix of protein from the animal, its feed and ruminal and intestinal bacteria.

Significance:

Characterization of faecal proteins has the potential to increase our understanding of host responses to changes such as diet, disease and drug-treatment. In-gel sample preparation prior to proteomics can be used to remove high molecular weight glycoproteins and reduce protein/peptide loss in FASP. This method of sample preparation will have application not only in the investigation of bovine faecal extracts but also in studies where large molecules such as glycoproteins or oligosaccharides could have detrimental influences on sample preparation involving ultrafiltration.

Graphical abstract:



Abstract:

Bovine faecal composition is complex and a knowledge gap exists in the understanding of the bovine faecal proteome. In the present study, in-gel sample preparation (IGSP) of faecal samples prior to proteomics showed an increase in the number of proteins identified in faecal samples compared to those processed by filter-aided sample preparation (FASP). The optimised sample preparation method removed high molecular weight glycoproteins as part of the clean-up process of the faecal samples, and in combination with in-gel digestion before liquid chromatography with tandem mass spectrometry (LC-MS/MS). The use of IGSP led to enhanced protein identification with increases in the number of peptides identified and in the percent coverage of proteins in the bovine faecal samples.

Keywords:

Bovine faeces; Mass spectrometry, In-gel sample preparation; Filter-aided sample preparation

4.1 Introduction

Filter-aided sample preparation (FASP) is a common proteomic sample preparation method for the generation of tryptic peptides prior to nano liquid chromatography and mass spectrometry (LC-MS/MS) (Wisniewski et al. 2009). However, in preliminary experiments we obtained an unexpectedly low number of protein identifications when using FASP to prepare bovine faecal samples for LC-MS/MS. We hypothesised that the low yield of known proteins was a consequence of the complexity of the sample matrix and the potentially extended period over which the matrix components were able to react. Faeces is a complex, heterogeneous, mixture of compounds that includes proteins from diet, host and microbiome, with a huge range of small, potentially chemically active molecules. Faecal samples comprise components from upper (proximal) and lower (distal) gastrointestinal tract, which have been allowed to interact with each other at body temperature for a period of time that could range from a few minutes in the case of rectal mucus, to a day or more, for the ingested feed components.

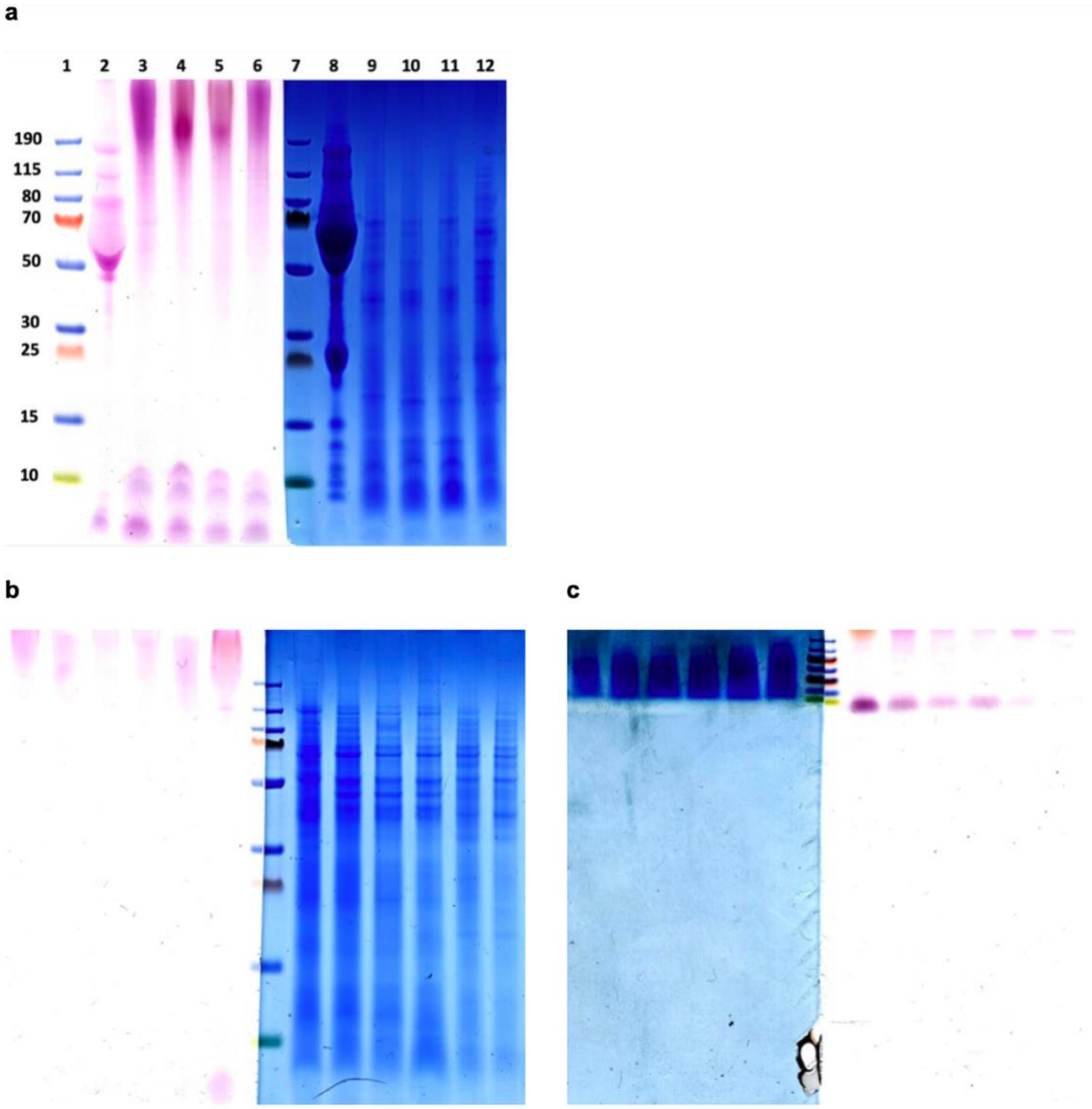
Modifications of the preparation procedures for faecal samples were therefore investigated to increase the protein identifications made by LC-MS/MS analysis. Possible causes of the low protein identification using FASP were also investigated. In assessing the protein composition of bovine faecal samples by SDS-PAGE, gels were stained with Coomassie blue for protein or with the periodic acid-Schiff (PAS) stain for glycoprotein. All bovine faecal samples were found to contain PAS-staining material with molecular weights (MW) greater than 200 kDa. The presence of these high MW glycoproteins could have had detrimental effects on the preparation of the faecal samples by FASP for proteomic analysis by LC-MS/MS. An in-gel sample preparation (IGSP) method to remove the high MW glycoproteins before trypsin digestion in the gel was developed and compared to FASP in terms of the number of protein identifications made following either of the preparation methods. The influence of gel pieces on digestion efficiency and peptide recovery was minimized by using the modified method from Goldman et al. (2019).

4.2 Methods and results

As part of an ongoing investigation, faecal samples were obtained from two groups of healthy beef cattle that were fed a diet composed of mostly barley cereal grains in which the barley had been treated with either ammonia or a preservative. Fresh faecal samples from each group were collected from the floor following observation of defaecation. Samples were refrigerated immediately after collection, transported directly to the laboratory, and stored at -80˚C until use. The method of faecal protein extraction was modified based on previous studies (Debyser et al. 2016, Zhang et al. 2018). Briefly, on thawing at room temperature, 3 g of faeces were mixed with 12 mL of sample buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, pH 7.4). One tablet of protease inhibitor (Roche Diagnostics, US) was added to every 25 mL of the sample buffer, followed by disruption by a homogenizer stomacher until there were no hard pellets remaining. The samples were centrifuged at 400 × g at 4 ˚C for 30 min, the supernatant was collected and sonicated on ice using an ultrasonic liquid processor (VC-130, Sonics & Materials, US) at 80% of amplitude for seven times of 5 s run interspersed with 10 s cool down. Samples were then centrifuged at 14,000 × g at 4 ˚C for 30 min. The supernatant was concentrated using an Amicon Ultra-15 centrifugal filter unit (10 kDa cut-off) (Merck & Co., US). The total concentration of protein of each sample was measured by the BCA method (Thermo Scientific, UK) with bovine serum albumin as standard. For this assessment of the benefit of IGSP, two faecal extracts (one from each group) were prepared by FASP and by IGSP, with results of protein identifications combined for each sample preparation method.

Periodic acid-Schiff staining is widely used in histochemistry and histological studies to show the presence of carbohydrates and carbohydrate-containing compounds. The presence of glycoproteins in a selection of the bovine faecal samples was determined by PAS stain method, modified from Segrest and Jackson (1972). Briefly, faecal samples, along with a bovine serum sample (from our previous study (Turk et al. 2021)) as control material, which has up to 50% of proteins being glycosylated (Fanayan et al. 2012), were loaded twice, on left- and right-hand sides of a 4-12% Bis-Tris gel (Invitrogen, UK) and run for 35 min at 200 V. Half of the gel was fixed in the fixative solution (40%, v/v ethanol with 5% v/v acetic acid) overnight and stained in the periodic acid solution (0.7% v/v periodic acid with 5% v/v acetic acid) for 2 h. Following washing in the sodium metabisulfite solution (0.2% w/v sodium metabisulfite with 5% v/v acetic acid) for 3 h (the solution was changed every 30 min), the gel was then stained in Schiff's fuchsin-sulfite reagent (Sigma, UK) overnight. The other half of the gel was stained in 0.1% w/v G250

Coomassie blue (Sigma, UK) for one hour and de-stained in 7.5% acetic acid with 20% methanol overnight (Figure 4-1a). Comparing the PAS and Coomassie blue-stained gels showed that the bovine faecal samples contained a high abundance of glycoproteins, the majority of which had MW higher than 190 kDa or lower than 10 kDa. This raised the possibility that an in-gel clean-up method could be used by excision of proteins within 10-190 kDa to exclude the highly abundant high MW glycoproteins from further proteomic analysis.



A bovine serum sample was run as references (lanes 2 and 8). Lanes 3 to 6 were loaded with the same bovine faecal samples as lanes 9 to 12 respectively.

Figure 4-1. (a) Samples in the 4-12% polyacrylamide gradient gel were stained by periodic acid-Schiff (left) and by Coomassie blue (right). Faecal samples were run on 10% Bis-Tris gels at 200 V for (b) 35 min and (c) 4 min, and were stained by Coomassie blue and Periodic acid-Schiff respectively.

The use of single percentage polyacrylamide gels (10%, Invitrogen, UK) was shown to better restrict entry of the high MW glycoproteins into the gel and help sample preparation. Running the gel for only a short time (4 min) before staining with Coomassie blue meant that all stained proteins could be included in a narrow gel section of 3-5 mm (Figure 4-1c) and enabled excision of all proteins from 10-190 kDa in this one gel piece (Goldman et al. 2019). This process therefore concentrated the proteins into a single band, eliminating the high MW glycoproteins from further processing and at the same time minimizing the gel

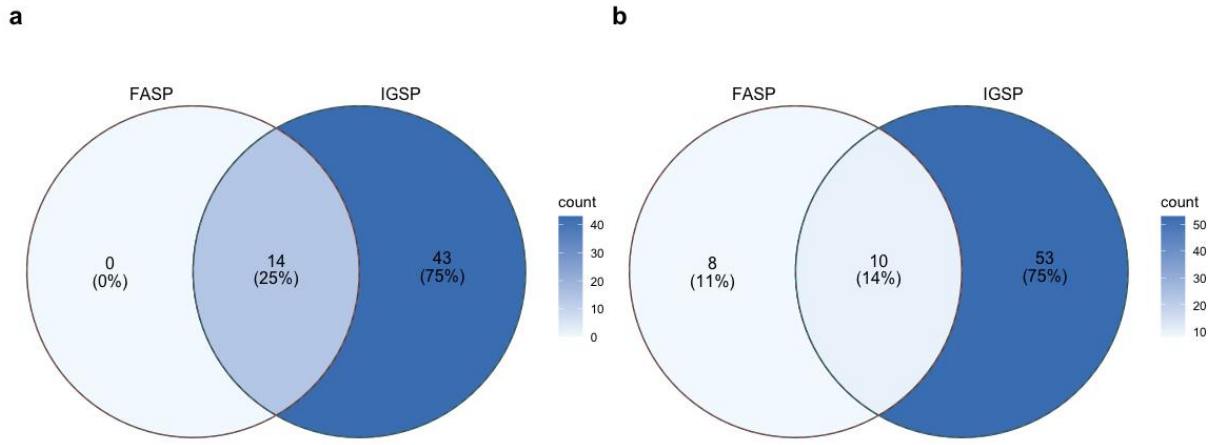
volume for in-gel digestion. Two bovine faecal samples were processed by IGSP (removal of the high MW glycoproteins in combination with in-gel digestion). Three replicates of each sample were run in separate tracks of the 10% polyacrylamide gels. The Coomassie blue-stained portions of each sample track of the gel were excised and processed by in-gel digestion, modified from Shevchenko et. al (2006). Briefly, excised gels were cut into small pieces with the gel pieces of the three replicates of each sample being pooled, followed by washing in 500 µL of 100 mM ammonium bicarbonate (ABC) and 500 µL of 50% of acetonitrile in 100 mM ABC for 30 min on a shaker, respectively. Samples were reduced in 10 µL of 45 mM dithiothreitol in 150 µL of 100 mM ABC at 60 ˚C for 30 min and were then alkylated in 20 µL of 100 mM iodoacetamide in the dark for 30 min. Samples were washed in 500 µL of 50% acetonitrile in 100 mM ABC for 30 min on a shaker, shrunk by acetonitrile and were then completely dried down in a vacuum centrifuge. Samples were incubated in 120 µL of 0.05 µg/µL trypsin in 25 mM ABC overnight. The supernatant was collected and the rest of the gel pieces were submerged in 40 µL of 5% formic acid for 20 min on a shaker, and incubated with 80 µL of 5% acetonitrile for another 20 min. The supernatant was pooled with the previously collected supernatant, and were dried down in a vacuum centrifuge. For comparison, the same two bovine faecal samples were processed by FASP method. One hundred µg of proteins from each sample were mixed with 5 µL of SDT-lysis buffer (4% SDS, 100 mM dithiothreitol (DTT), 100 mM Tris-HCl, pH 7.6) and were moved to the filter unit (10,000 MWCO, Expedeon, UK). Two hundred µL of urea buffer (8 M urea in 100 mM Tris-HCl, pH 8.5) were added to the samples and were centrifuged at 14,000 × g for 15 min (repeated). Following an addition of 100 µL of 50 mM iodoacetamide (in urea buffer), samples were incubated in darkness for 20 mins. Samples were centrifuged at 14,000 × g for 10 min and the flow-through was discarded. Samples were washed with 100 µL of urea buffer and 100 µL of 50 mM ABC three times respectively, for 15 min each at 14,000 × g. Each sample was digested by 1 µg of trypsin (in 50 mM ABC) overnight at 37 ˚C. The filter units with digested samples were transferred into new eppendorfs and samples were collected by centrifuge (14,000 × g for 10 min). Fifty µL of 10% acetonitrile were added to each sample and centrifuged at 14,000 × g for 10 min, the flow-through was collected and pooled with the previously collected digested samples. Samples were mixed with 1 µL of 1 % trifluoroacetic acid, and were dried down in a vacuum centrifuge. Five µg of peptide samples prepared from both methods were analysed by a nanoflow ultrahigh-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (nUHPLC-ESI-MS/MS). The peptides were solubilized in 20 µL 5% acetonitrile with 0.5% formic acid using the auto-sampler of a nanoflow uHPLC system (RSLCnano, Thermo Scientific, UK)

and were detected online by ESI-MS with an Orbitrap Elite MS (Thermo Scientific, UK). Ionisation of LC eluent was performed by interfacing the LC coupling device to an NanoMate Triversa (Advion Bioscience) with an electrospray voltage of 1.7 kV. An injection volume of 5 µL of the reconstituted protein digest was desalted and concentrated for 10 min on a C18 trap column (Acclaim PepMap C18 100Å 0.3 × 5 mm, 5µM particle size, Thermo Scientific, UK) using a flow rate of 25 µL/min with 1% acetonitrile with 0.1% formic acid. Peptide separation was performed on an Acclaim PepMap C18 100A° phase column (50 cm × 75 µm, particle size 3 µm, Thermo Scientific, UK) using a solvent gradient at a fixed solvent flow rate of 0.3 µL/min for analytical column. The solvent composition was (A) 0.1% formic acid and (B) 0.08% formic acid in 80% acetonitrile. The solvent gradient was 4% B for 12 min, 4 to 60% B for 90 min, 60 to 99% B for 14 min and held at 99% B for 5 min. A further 9 min at initial conditions for column re-equilibration was used before the next injection. The Orbitrap Elite acquires a full-scan MS in the range 300 to 2,000 m/z for a high-resolution precursor scan at 60,000 RP (at 400 m/z), while simultaneously acquiring up to the top 15 precursors which are isolated at 0.7 m/z width and subjected to CID fragmentation (35% NCE) in the linear ion trap using rapid scan mode. Singly charged ions are excluded from selection, while selected precursors are added to a dynamic exclusion list for 30 s.

Raw data generated by LC-MS/MS were imported into Proteome Discoverer (version 2.4, Thermo Scientific, UK). Faecal samples contain proteins from the animal, from the plant-based diet (mainly barley plus other species) and microorganisms ingested with the diet or resident in the gastrointestinal tract. Therefore, the data were assessed using Sequest HT engine to interrogate sequences in the Swissprot databases of bovine, barley and bacterial proteins. For the latter the database search focused on known ruminal and faecal microorganisms (*Ruminococcaceae, Lachnospiraceae, Clostridiaceae, Prevotellaceae, Bacterioidaceae, Spirochaetaceae*) (Holman and Gzyl 2019). Precursor mass tolerance was set as 10 ppm and the fragment mass tolerance as 0.6 Da. Carbamidomethylation of cysteine was specified as fixed modification, and oxidation of methionine, deamidation of asparagine/glutamine and acetylation of lysine and N-term were set as dynamic modifications. Data organization and graphing (package *ggVennDiagram*) were performed in R software (version 4.0.3) (Team 2020).

Comparisons between the use of FASP and IGSP for the identified bovine and bacterial proteins (master proteins with at least two unique peptides) are shown in Venn diagrams (Figure 4-2), and comparisons for all the master proteins are shown in Venn diagrams in

Appendix Figure 4-1. Data for analysis combined results of the two samples that were either processed by FASP or IGSP method. For each of the databases interrogated, more proteins were identified using the IGSP method than the FASP method. For the identified master proteins with at least two unique peptides, the number of bovine proteins increased around four-fold from 14 with FASP to 57 by IGSP, while increasing similarly from 18 to 63 proteins for the bacterial proteins identified. For the barley proteins in the faeces, only serpin-Z4, serpin-Z7 and alpha-amylase/trypsin inhibitor CMb were found in samples that were digested by FASP. In comparison, another five barley proteins (alpha-amylase/trypsin inhibitor CMd, alpha-amylase inhibitor BDAI-1, alpha-amylase inhibitor BMAI-1, phytepsin and signal recognition particle 54 kDa protein 3) were found in the samples that were prepared by IGSP. As examples of these findings, Table 4-1 shows a selection of 38 identified proteins separated by origin (barley, bovine or bacteria) and by presence in samples prepared by FASP or IGSP. The proteins being selected on the basis of the number of peptides identified in the IGSP groups. It was noticeable that with IGSP, not only were more proteins identified but that for proteins identified when prepared by both methods, the number of peptides and % coverage for each protein was greater in samples prepared by IGSP. For example for the barley protein serpin-Z4, the number of peptides increased from 4 to 7 and coverage increased from 11% to 25%, for bovine protein alpha-2 macroglobulin, the number of peptides increased from 5 to 28 with the % coverage increasing from 4% to 26% and for bacterial protein phosphoenolpyruvate carboxykinase (*Agathobacter rectalis*), the number of peptides increased from 3 to 9 with the % coverage increasing from 7% to 24%. Of the 19 proteins of Table 4-1 that were identified in proteins when prepared by both FASP and IGSP, the number of peptides increased by an average of 2.9 times and the coverage by an average of 3.5 times when IGSP was used. The full list of proteins with at least two peptides identified in each of the two methods is given in Appendix Table 4-1.



Proteins assessed here were the master proteins that had at least two unique peptides identified.

Figure 4-2. Comparisons between filter-aided sample preparation (FASP) and in-gel sample preparation (IGSP) in the bovine faecal sample identifications: (a) bovine proteins and (b) bacterial proteins (*Ruminococcaceae, Lachnospiraceae, Clostridiaceae, Prevotellacea, Bacterioidaceae, Spirochaetaceae*).

Table 4-1. The improvements of in-gel sample preparation (IGSP) in faecal protein identifications (peptide coverage percentage and numbers) from bovine, barley and bacteria databases compared to filter-aided sample preparation (FASP).

Gene name	Protein	FASP		IGSP	
		Coverage (%)	Peptides	Coverage (%)	Peptides
Barley: in FASP and IGSP					
IAT2	Alpha-amylase/trypsin inhibitor CMb	18	2	28	3
PAZ1	Serpin-Z4	11	4	25	7
PAZ7	Serpin-Z7	8	3	11	4
Barley: only in IGSP					
IAT3	Alpha-amylase/trypsin inhibitor CMd	-	-	40	4
IAD1	Alpha-amylase inhibitor BDAI-1	-	-	30	3
Bovine: in FASP and IGSP					
ALB	Albumin	12	8	57	29
MPTX	Mucosal pentraxin	17	4	49	8
ANXA4	Annexin A4	10	3	34	9
ENPP3	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	5	4	30	16
A2M	Alpha-2-macroglobulin	4	5	26	28
SERPINA1	Alpha-1-antiproteinase	5	2	25	8
ANPEP	Aminopeptidase N	2	2	10	8
C3	Complement C3	3	5	9	12
Bovine: only in IGSP					
LYZ3	Lysozyme C-3	-	-	61	6
S100A9	Protein S100-A9	-	-	41	4
PIGR	Polymeric immunoglobulin receptor	-	-	28	13
ALPI	Intestinal-type alkaline phosphatase	-	-	28	9
SERPINA3-1	Serpin A3-1	-	-	23	8
DPEP1	Dipeptidase 1	-	-	20	6
LTF	Lactotransferrin	-	-	18	9

DPP4	Dipeptidyl peptidase 4	-	-	15	9
Bacteria: in FASP and IGSP					
pckA	Phosphoenolpyruvate carboxykinase (ATP) (*Lachnospira eligens*)	8	3	31	10
tuf	Elongation factor Tu (*Agathobacter rectalis*)	6	2	26	7
pckA	Phosphoenolpyruvate carboxykinase (ATP) (*Agathobacter rectalis*)	7	3	24	9
ilvC	Ketol-acid reductoisomerase (NADP(+)) (*Clostridium botulinum*)	13	4	22	6
tuf1	Elongation factor Tu (*Clostridium perfringens*)	6	2	19	5
fusA	Elongation factor G (Lachnoclostridium phytofermentans)	4	2	14	9
argC	NAD(P)-specific glutamate dehydrogenase (*Prevotella ruminicola*)	5	2	12	4
pnp	Polyribonucleotide nucleotidyltransferase (*Bacteroides thetaiotaomicron*)	3	2	7	4
Bacteria: only in FASP					
rpsM	30S ribosomal protein S13 (*Bacteroides thetaiotaomicron*)	14	2	-	-
gpmI	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*Bacteroides v*ulgatus)	4	3	-	-
pfp	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase (*Spirochaeta thermophila*)	4	2	-	-
tcdA	Toxin A (Clostridium novyi)	1	2	-	-

Bacteria: only in IGSP					
gdh	NAD-specific glutamate dehydrogenase (*Clostridium symbiosum*)	-	-	32	11
pckA	Phosphoenolpyruvate carboxykinase (ATP) (*Bacteroides fragilis*)	-	-	10	5
ppdK	Pyruvate, phosphate dikinase (*Clostridium symbiosum*)	-	-	9	6
fucI	L-fucose isomerase (Bacteroides thetaiotaomicron)	-	-	9	5

Coverage is calculated by dividing the number of amino acids in all identified peptides by the total number of amino acids in the entire protein sequence.

4.3 Discussion and conclusion

There were four bacterial proteins shown in Table 4-1 that were only found in samples prepared by FASP. These proteins could have been partially digested to peptides in the intestine but held by non-covalent, protein-protein interaction in large molecular complexes which would be retained by the filter in FASP. In contrast, in the IGSP the complexes would be broken down by the presence of SDS and reducing agent and the released peptides, migrating with the dye front in SDS-PAGE would not be included in the gel piece excised prior to trypsinisation. The loss of identification of such proteins is a limitation of the method but greatly outweighed by the many more proteins identified with IGSP.

In-gel sample preparation, by running samples for a short-distance in the polyacrylamide gel and excising the entire protein staining bands prior to in-gel digestion (Beer et al. 2017), combined with bottom-up proteomics analysis, had been reported to identify with a large number of high-confidence peptides and proteins in human cell lines (Gholami et al. 2013), and to increase the depth of analysis of plasma samples, which have a large dynamic range of protein abundances. In the present study, the IGSP method not only avoided the influence of MS-incompatible detergents, buffers or salts (Goldman et al. 2019), but also removed high-abundance and high MW glycoproteins that may affect the identification of lower-abundance proteins. The recognition of the role of high MW glycoproteins in poor protein identification using the FASP method and the ability of IGSP to overcome this problem can contribute to proteomic investigation of faecal samples and other samples where this might occur. Although this study compared IGSP to FASP in the preparation of two samples, the use of IGSP has consistently given a greater yield of protein identification when used for preparation of multiple faecal extract samples for quantitative proteomic study.

The high MW glycoproteins are probably intestinal mucins, but remain to be identified, and could have detrimental effects on the use of FASP in at least two ways. The presence of such large molecules can block the pores in the filtration devices used such that the efficiency of filtration is greatly reduced leading to loss of trypsin-digested peptides for MS analysis. Furthermore, mucin has an inhibitory effect on protein digestion by trypsin as one of its important roles in the intestine is to resist endogenous proteases such as trypsin in order to protect the intestine while food is digested (Johansson M.E.V et al. 2013). Mucins remain intact in the presence of digestive enzymes so will have a similar effect on

the trypsin used in FASP. Removal of the mucin by use of IGSP overcomes both obstacles to the proteomic study of bovine faecal samples. The identification of plant-based diet (barley) proteins in the faeces provided new areas for the study of animal digestion and absorption. Interestingly, the serine protease inhibitors identified in bovine faecal samples, serpin-Z4 and serpin-Z7 also survive through malting and brewing in the beer making process, and are proteins found in beer froth (Evans et al. 1999, Iimure et al. 2010). The effects of plant-derived protease inhibitors on protein digestibility have aroused interest in human gastrointestinal health studies (Karlund et al. 2021). In conclusion, the use of IGSP in proteomics improved protein identification in bovine faeces compared to proteomics based on FASP method and could benefit future studies in quantitative protein investigations of bovine faeces.

4.4 Author contribution

The experiments were done by myself, with the help from Glasgow Polyomics. I was also responsible for the data analyses, visualisation and thesis writing.

Chapter 5 A Mixture of Host, Dietary and Microbial Proteins in Bovine Faeces Revealed by TMT-based Proteomics

5.1 Introduction

Faeces is a complex, heterogeneous, mixture of compounds from host, diet, and microbiota, including a huge range of small and potentially chemically active molecules. Studies of faeces are useful in understanding gastrointestinal tract (GIT) pathology, medical diagnosis and prognosis, offering potential for finding disease biomarkers (Ang et al. 2017), and drug development (Jin et al. 2017). The colour, odour, shape, and consistency of faeces provides information about the state of the host: (Gu et al. 2015, O' Reilly et al. 2021, Huang et al. 2022) dietary information (Sistiaga et al. 2014), behaviour and physiology (Tsutaya et al. 2021), and the interactions between the host and the microbiota (Lichtman et al. 2015). As described in Chapter 1, collection of faecal samples can be easy and non-invasive, allowing repeated sampling. For the diagnosis of GI diseases, faeces has been recommended as an ideal alternative to blood (Ang et al., 2017), because some specific components in the faeces that are derived from GI tumours or other epithelial lesions might be present at relatively higher concentrations than those in blood or urine due to the close proximity of sampling to the lesion, and lack of dilution by circulation in blood or lymphatics.

In human clinical practice, faecal proteomics has primarily been applied in the diagnosis of infections, poor nutrient digestion and absorption, as well as in cancer diagnosis. Researchers have found potentially useful faecal proteins in human diseases, including inflammatory bowel disease (Roseth et al. 1996, Lehmann et al. 2015), colorectal cancer (Ang and Nice 2010, Ang et al. 2011, Bosch et al. 2017) and cystic fibrosis (Debyser et al. 2016). Faecal proteomes in mouse (Ang et al. 2010), monkey (Tsutaya et al. 2021), dog (Cerquetella et al. 2019, O' Reilly et al. 2021) and sheep (Palomba et al. 2018) have also been studied. However, much of the research on bovine faeces has focused on the microbiota, while the host and dietary proteins present in faeces remain to be investigated. Label-free liquid chromatography with tandem mass spectrometry (LC-MS/MS) of sample solution or of specific in-gel digested proteins have been the most commonly used approaches in such studies. Multiplex isobaric labelling approaches, including tandem

mass tagging (TMT), enable qualitative and quantitative analysis of multiple sample protein groups at the same time and reduce sample handling and inter-analysis variability, and thus TMT was applied in the present study.

From our previous studies described in Chapter 3, finishing beef cattle fed on diets in which the cereal grains were treated with ammonia to conserve the feed with an elevated pH, outperformed cattle fed cereal grains without the treatment (Huang et al. 2022), or treated with propionate. The faecal starch concentration and proportion of animals with diarrhoea among the animals that were fed ammonia-treated barley-based diet were lower than in the animals fed propionate-treated barley-based diet. We wished to follow up on the apparent differences in faecal starch and diarrhoea incidence with a proteomic investigation but with the initial step to refine the methods of sample processing to improve rates of protein identification as described in Huang et al. (2022).

Having developed satisfactory protocols, as described in Chapter 4, we aimed to apply TMT-based proteomics (1) to characterise the bovine, barley and microbial proteomes in the bovine faeces, (2) to identify the most abundant proteins from host, diet, and microbiome and (3) to determine whether the dietary treatment was associated with differential protein abundance in the faeces. The results were expected to provide baseline information about the relative abundance of host, dietary and microbial proteins in bovine faeces and will inform the development of our future studies to optimize diets for cattle.

5.2 Materials and methods

5.2.1 Sample collection and protein extraction

The present study used samples collected in a previous study (Jonsson et al. 2018), which was carried out on a commercial beef breeding and finishing unit in Aberdeenshire, in northeast Scotland. More details can be found in Chapter 3, in which it is referred to as Trial 1. Four pens of cattle were given one of two diets: ammonia-treated barley-based diet (ATB, 93 cattle) or propionate-treated barley-based diet (PTB, 124 cattle). Five to ten fresh faecal samples from each pen were collected approximately every two weeks from the floor following observation of defaecation from 07:30 h of the day of sampling. Faeces were scored from 1 to 5 according to their consistency, with 1 being very dry and forming a pile of more than 50 mm high, and 5 being moist to liquid with blood or mucus. Samples were refrigerated (4 ˚C) immediately after collection, transported directly to the laboratory, and stored at -80 ˚C until use. For this study, to reduce potential confounding due to variation in faecal dry matter content and rate of passage through the GIT, we selected the five highest volume faecal samples that were scored either 1 or 2 (i.e., from cattle without diarrhoea) from each group from the collection on D-81.

The protein extraction method was modified from previous studies (Debyser et al. 2016, Zhang et al. 2018, Huang et al. 2022). After thawing on ice, 0.5 g of faeces was mixed with 1 mL of buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, pH 7.4) by bead-beating using Lysing Matrix E 2 mL tubes which contain 1.4 mm ceramic spheres, 0.1 mm silica spheres and one 4 mm glass bead (MP Biomedicals, UK). One mini protease inhibitor cocktail tablet (Sigma, UK) was added to every 7 mL of the buffer. Three replicates per sample were processed and were bead-beaten at 6.5 m/s for 45 s interspersed with 3 min cool-down, three times in total until there were no hard pellets remaining. Following centrifugation at 1,000 × g for 5 min at 4 ˚C, supernatant was collected, and the three replicates of each sample were pooled. The process of bead beating was repeated twice. The supernatant was sonicated (VC-130 Ultrasonic liquid processor, Sonics & Materials, US) on ice at 80 % of amplitude for five times of 5 s run interspersed with 10 s cool down, followed by centrifuging at 14,000 × g for 30 min at 4 ˚C. The supernatant was concentrated on an Amicon Ultra-15 centrifugal filter unit with 10 kDa cut-off (Merck & Co., US), centrifuging at 3,200 × g for 30 min at 4 ˚C, re-diluted to the starting volume with buffer without SDS and protease inhibitor, and the process repeated three times. The total concentration of proteins of each ample was measured by the BCA method (Thermo

Scientific, UK) with bovine serum albumin as standard. Results of faecal protein extraction was demonstrated by SDS-PAGE: thirty µg of each faecal protein sample was run on a 10 % Bis-Tris gel (Invitrogen, UK) at 150 V for 80 min; the gel was stained in 0.1% w/v G250 Coomassie blue (Sigma, UK) for one hour and de-stained in 7.5% acetic acid with 20% methanol overnight.

5.2.2 TMT labelling and LC-MS/MS

The faecal samples were processed further by an in-gel sample preparation (IGSP) method to remove high MW glycoprotein using a short (5 min) run on SDS-PAGE using 10% polyacrylamide gels. Proteins were digested in-gel by porcine trypsin (Thermo Scientific, UK) to obtain peptides for labelling with TMT conjugates. Details have been described in Chapter 4 (Huang et al. 2022). The TMT 10plex label reagents (UA280170, Thermo Scientific, UK) were equilibrated at room temperature. Forty-one µL of anhydrous acetonitrile were added to the vials and mixed thoroughly. Twenty µg of each peptide sample was incubated in 8.2 µL of individual TMT label reagent, respectively, for one hour at room temperature, followed by addition of 1.6 µL of 5 % hydroxylamine and incubated for 45 min. For each sample, 0.6 µg were taken, and the ten TMT labelled samples were pooled for nanoflow ultrahigh-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (nUHPLC-ESI-MS/MS).

The systems of nUHPLC-ESI-MS/MS were similar to that previously described (Huang et al. 2022), except that the sample was desalted and concentrated for 12 min on the trap column; the solvent gradient was 4 % of 0.08 % formic acid in 80 % acetonitrile (B) for 10 min, 4 to 60 % B for 170 min, 60 to 99 % B for 15 min, held at 99 % B for 5 min; a further 10 minutes at initial conditions for column re-equilibration was used before the next injection. The Orbitrap Elite MS cycled through acquisition of a high-resolution precursor scan at 60,000 RP (over a mass range of 380 – 1,800 m/z) followed by isolation and CID fragmentation the top 3 precursor ions from the MS scan in the linear ion trap. The three precursor ions were also subjected to HCD in the HCD collision cell followed by detection in the Orbitrap, to release TMT reporter ions. Singly charged ions were excluded from selection, while selected precursors were added to a dynamic exclusion list for 180 s.

5.2.3 Protein identification

Protein identification and relative quantification were performed in Proteome Discoverer software (PD, version 2.4, Thermo Scientific, UK). The data were assigned using Sequest

HT engine to interrogate sequences in the Swissprot *Bos taurus* (cattle) and *Hordeum vulgare* (barley) databases, for identification of host and diet proteins, respectively. The TrEMBL databases and other subgenera in *Bos* and *Hordeum* were also used to augment the incomplete Swissprot databases. The databases were downloaded on 13/03/2022, and the database for the host protein identification consisted of 161,320 sequences in total and for the dietary proteins consisted of 211,400 sequences. The identification of bacterial proteins was focused on the databases of five genera (SwissProt and TrEMBL, 3,655,253 sequences), *Clostridium*, *Bacteroides*, *Ruminococcus*, *Prevotella* and *Eubacterium*, which were previously reported in faeces (Holman and Gzyl 2019), and the identification of archaeal proteins was focused on methanogenic genera (including 30 genera in *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanopyrales*; SwissProt and TrEMBL, 581,222 sequences). Two trypsin missed cleavage sites were allowed, the threshold of precursor mass tolerance was set at 10 ppm, and the fragment mass tolerance was set at 0.6 Da. Carbamidomethylation of cysteine was set as a fixed modification, and TMT 6plex of lysine and peptide N-terminus, oxidation of methionine, deamidation of asparagine/glutamine and acetylation of lysine and N-term were set as dynamic modifications. False discovery rates were at the most 1 %. Only proteins with at least two unique peptides matching the databases were considered as confidently identified proteins.

5.2.4 Validation of proteomics by western blot

The faecal samples were subjected to western blot analysis of two candidate proteins making use of antibodies that were available for use – an antibody to bovine serum albumin, already conjugated to horse radish peroxidase (HRP) and an antibody to barley serpin Z4, described in Tanner et al (2019) to validate the results of the proteomics. Polyacrylamide gels (10 %, Invitrogen, UK) were run at 150 V for 80 min, with 30 µg of each faecal protein per lane for albumin and 50 µg of faecal protein for serpin Z4. One µg of bovine serum was used as control material in western blot of albumin; for serpin Z4, 400 ng of barley protein was used as control, and for further information 200 ng and 400 ng of protein from a Scottish barley-based beer (Original Best, Belhaven, UK) were also tested, as barley serpins are present in beer (Colgrave et al. 2012). Barley protein extraction was achieved using a mortar and pestle, then ultrasonication, dissolving the protein in the same buffer as used for the faecal samples, followed by washing and concentrating. The gels were rinsed in water and blotted to nitrocellulose transfer membranes (Invitrogen, UK) using an iBlot^{TM} gel transfer device (Invitrogen, UK). The

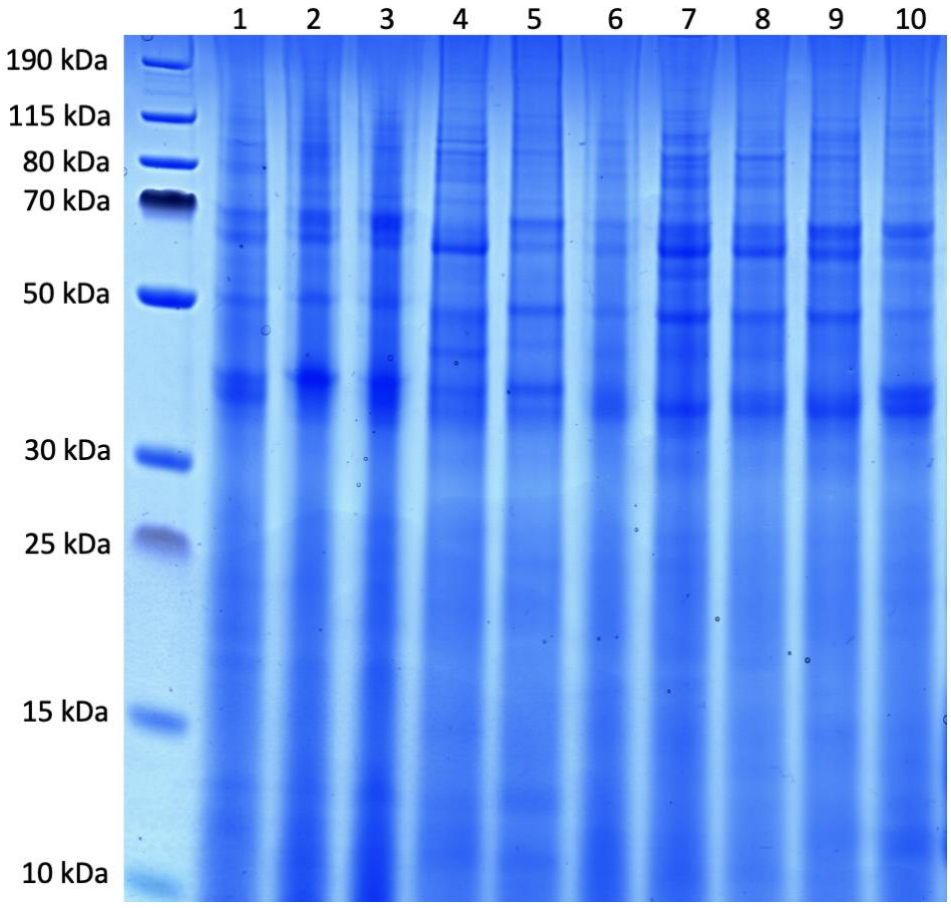
membranes were stained by Ponceau S to provide a visual assessment of protein loading in each lane, followed by washing with Tris-buffered saline (TBST) for 15 min three times. The membranes were blocked by 5 % skimmed milk powder in 0.1 % Tween 20 in TBST for 1.5 h. Rabbit polyclonal anti-bovine serum albumin-HRP (antibodies-online GmbH, Germany) was added at ratio of 1:1,000 and rabbit polyclonal anti-serpin Z4, a gift from Prof Greg Tanner (Tanner et al. 2019), was added at ratio of 1:2,000 with incubation overnight at 4 ˚C. The membranes were washed with TBST for 15 min three times. For albumin, the complexes were detected by ECL (Thermo Scientific, UK) and visualized using radiographic film (Hyperfilm ECL, Amersham Biosciences) after this wash, while the membrane for serpin Z4 was incubated with 1: 3,000 of goat anti-rabbit IgG HRP (Abcam, US) for 1 h before three-time TBST washes, ECL reaction and visualization.

5.2.5 Data analysis

Statistical analyses and result imaging were mainly performed in R version 4.0.3 (R Core Team, 2020). Protein abundance differences between the two diets were calculated using the pairwise ratio; the hypothesis test was background-based *t*-test in the PD software. The criteria for differential abundance of proteins were fold change (FC) of ATB to PTB > 1.5 or fold change of PTB to ATB > 1.5 and $p < 0.05$. Except for the built-in analysis in PD, gene-enrichment and functional annotation analysis of bovine and barley proteins were processed in STRING (https://string-db.org) and DAVID (https://david.ncifcrf.gov) and for bacterial and archaeal proteins it was processed in Unipept (Mesuere et al. 2016) (https://unipept.ugent.be). The analysis of protein band intensity of western blot was quantified using Image J software, and the differences in proteins between groups were tested using Mann-Whitney-Wilcoxon tests. The relationship between the intensity of protein bands in western blot and the relative abundance detected by proteomics was estimated by the Spearman correlation coefficient.

5.3 Results

The faecal samples used in the present study were scored either 1 or 2 for consistency, meaning that the samples were from cattle that had well-digested fibre and no diarrhoea. Clear protein bands could be seen from the Coomassie blue-stained gel (Figure 5-1), which showed successful extraction of proteins from all samples. The protein bands in the range of 30 kDa - 190 kDa were clear and sharp, where high abundance proteins were expected but there were no clearly differentiated protein band patterns between ATB and PTB groups.



Lanes 1-5 were samples from ATB group and lanes 6-10 were samples from PTB group.

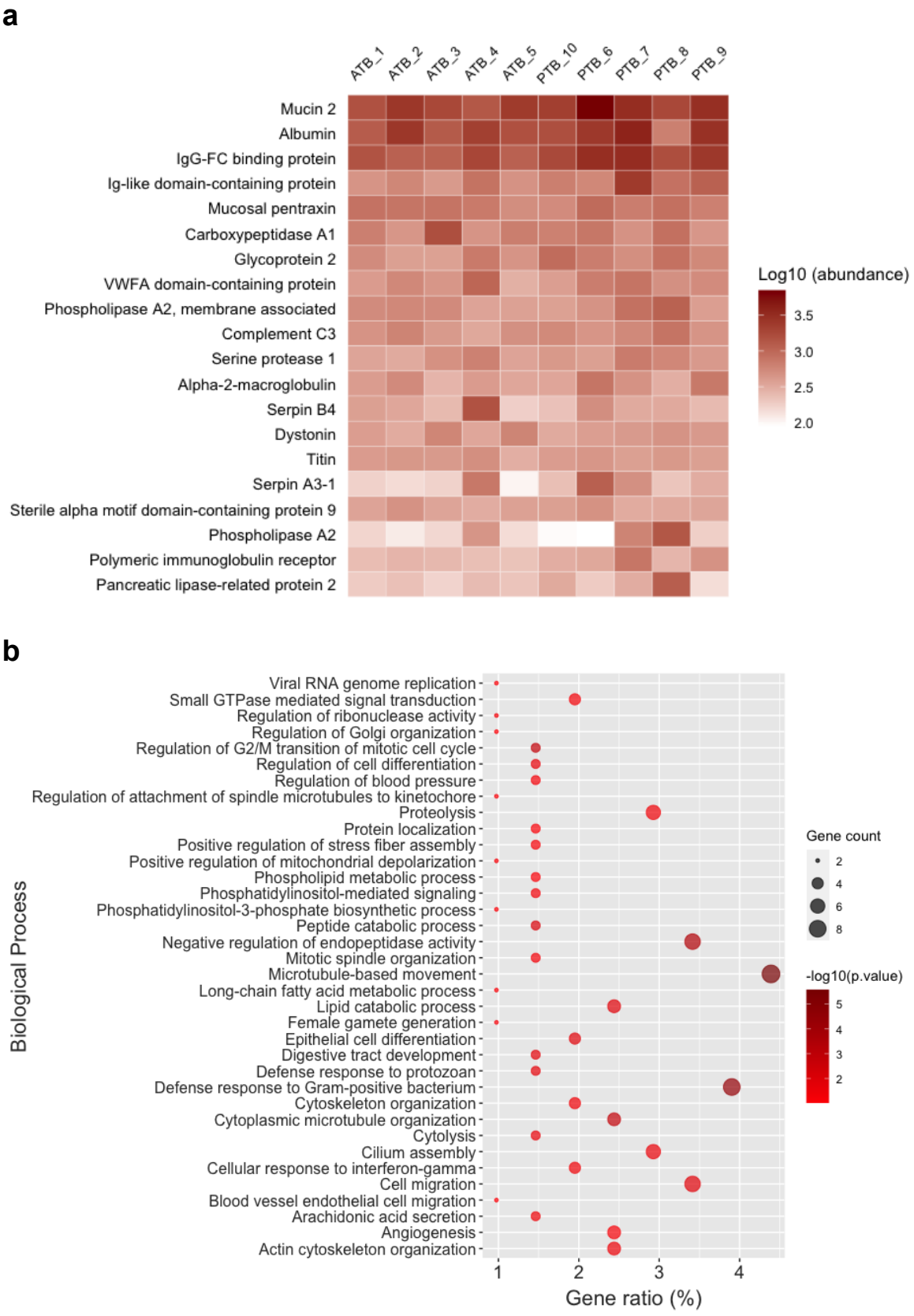
Figure 5-1. Bovine faecal proteins were shown on a 10% Bis-Tris gel by Coomassie blue staining.

5.3.1 Proteomics

In total, 281 bovine proteins, 199 barley proteins, 176 bacterial proteins and 190 archaeal proteins were identified in the bovine faeces and were used for further data analyses. The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2022) partner repository with the data set identifier PXD036027.

5.3.1.1 Host proteins

The 20 most abundant bovine proteins are listed in Figure 5-2 a. Among the top 20 bovine proteins, there were: glycoproteins (mucin-2 and glycoprotein 2), enzymes (carboxypeptidase A1, serine protease 1 and phospholipase A2), proteins that inhibit protease activities including alpha-2-macroglobulin and serpin A3-1, and proteins that are involved in inflammation including complement C3 and polymeric immunoglobulin receptor. Based on gene-enrichment and functional annotation analysis (Figure 5-2 b), host proteins identified in the faeces were significantly over-represented in biological processes including microtubule-based movement, defence response to Gram-positive bacterium, negative regulation of endopeptidase activity, cell migration and proteolysis. Proteins that are involved in catabolism of lipid and development of digestive tract were also identified.



P-values are EASE scores in DAVID, which are modified Fisher exact *p*-values; gene ratio (%) represents the percentage of the identified genes in the total genes of the given process.

Figure 5-2. (a) The 20 most abundant host proteins in the faeces, and (b) gene-enrichment and functional annotation analysis of the host proteins.

5.3.1.2 Barley proteins

One hundred and ninety-nine barley proteins were identified in the faeces. Serpin Z4 was the most abundant barley protein identified; serpin Z7 was identified in the samples as well. Barley proteins including actin, elongation factor Tu, elongation factor 1-alpha and dentin sialophosphoprotein-like protein were also among the top 20 abundant proteins (Figure 5-3). Some barley enzymes were present in the bovine faeces, including telomerase reverse transcriptase, E1 ubiquitin-activating enzyme, peroxidase, hexosyltransferase, E3 ubiquitin ligase, acetyl-CoA carboxylase and 1,3-beta-glucan synthase. The barley proteins in the faeces were related to binding histone, macromolecular complex and ATP - more information is shown in Table 5-1.

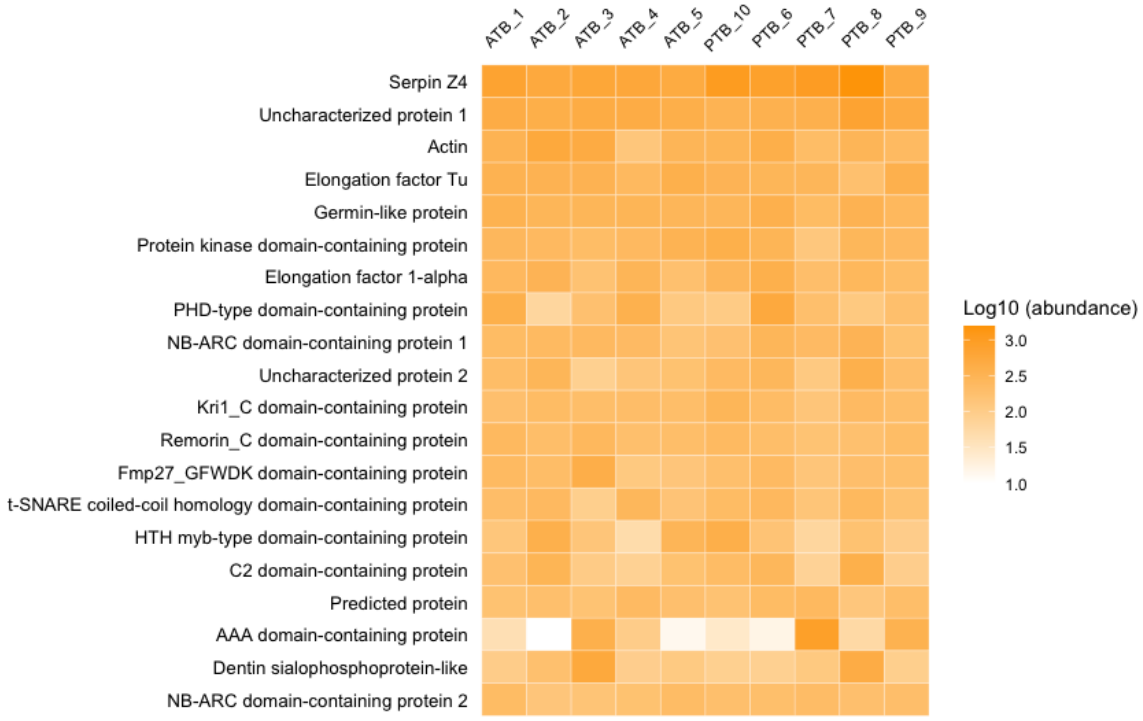


Figure 5-3. The 20 most abundant barley proteins identified in the bovine faeces.

Table 5-1. Molecular functional enrichment of the barley proteins identified in the faeces.

Term ID	Description	Observation	Background	Strength	FDR
GO:0042393	Histone binding	6	148	1.04	0.0045
GO:0044877	Protein-containing complex binding	7	305	0.79	0.0207
GO:0005524	ATP binding	35	4210	0.35	0.0018
GO:0035639	Purine ribonucleoside triphosphate binding	36	4503	0.33	0.0019
GO:0032555	Purine ribonucleotide binding	36	4561	0.33	0.002
GO:0005515	Protein binding	27	3376	0.33	0.0148
GO:0043168	Anion binding	39	5468	0.29	0.0048
GO:0036094	Small molecule binding	37	5399	0.27	0.0148
GO:0097159	Organic cyclic compound binding	64	10105	0.23	0.00085
GO:1901363	Heterocyclic compound binding	64	10083	0.23	0.00085
GO:0005488	Binding	92	16636	0.17	0.00019

Observation and Background represent gene count of observation and background, respectively; strength is the natural-log-transformation of the ratio between observed gene count and expected gene count (the number of proteins that were expected to be annotated with the term in a random network of the same size). FDR (false discovery rate) was *p*-value corrected for multiple testing within each category using Benjamini-Hochberg.

5.3.1.3 Microbial proteins

In total, 176 bacterial proteins were identified from genera *Clostridium* (91), *Prevotella* (46), *Bacteroides* (17), *Ruminococcus* (14) and *Eubacterium* (8). The 20 most abundant bacterial proteins are listed in Figure 5-4 a. The bacterial proteins in bovine faeces were over-represented in biological processes including gluconeogenesis; glycolysis; cellular amino acid metabolic process; glucose metabolism and translation. Figure 5-4 b shows 20 biological processes that most of the identified bacterial proteins (peptides) involved in (a full list of biological processes with at least 2 peptides involved can be found in the Appendix Table 5-1).

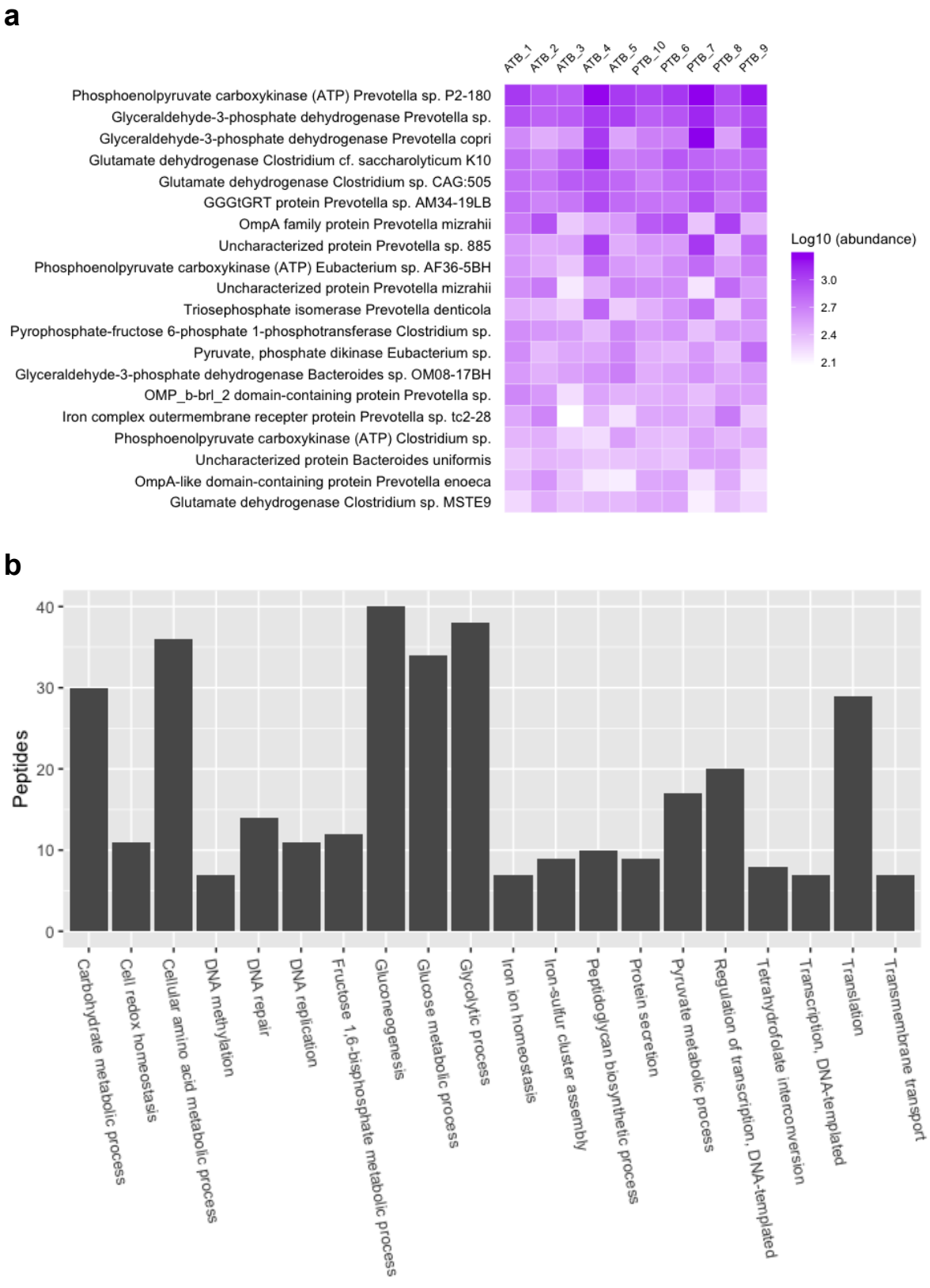


Figure 5-4. (a) The 20 most abundant bacterial proteins identified in the bovine faeces; and (b) the 20 biological processes in which most faecal bacterial peptides were involved.

One hundred and ninety archaeal methanogenic proteins were identified in the faecal samples, a large proportion of which belonged to *Methanobrevibacter* (50), followed by *Methanosarcina* (31), *Methanobacterium* (21), *Methanosphaera* (12), *Methanolobus* (10), with fewer than 10 proteins from each of the other searched genera. The 20 most abundant archaeal proteins are listed in Figure 5-5 a. The proteins were over-represented in carbohydrate metabolism; translation; regulation of transcription, DNA-templated; DNA repair, replication and recombination; and transmembrane transport. The 20 biological processes in which the most faecal archaeal peptides were involved are shown in Figure 5-5 b. Full list of biological processes with at least 2 peptides involved can also be found in the Appendix Table 5-1.

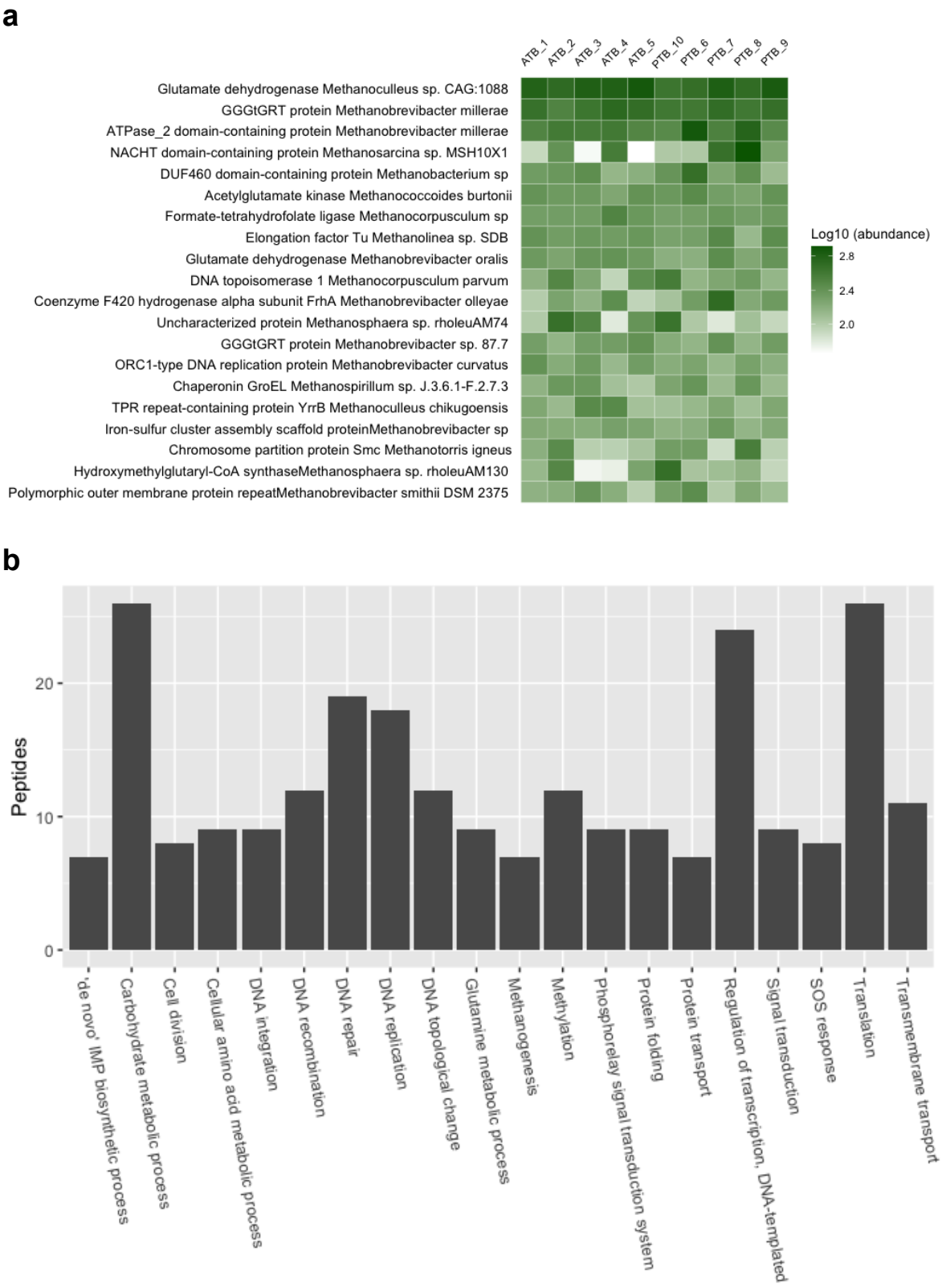
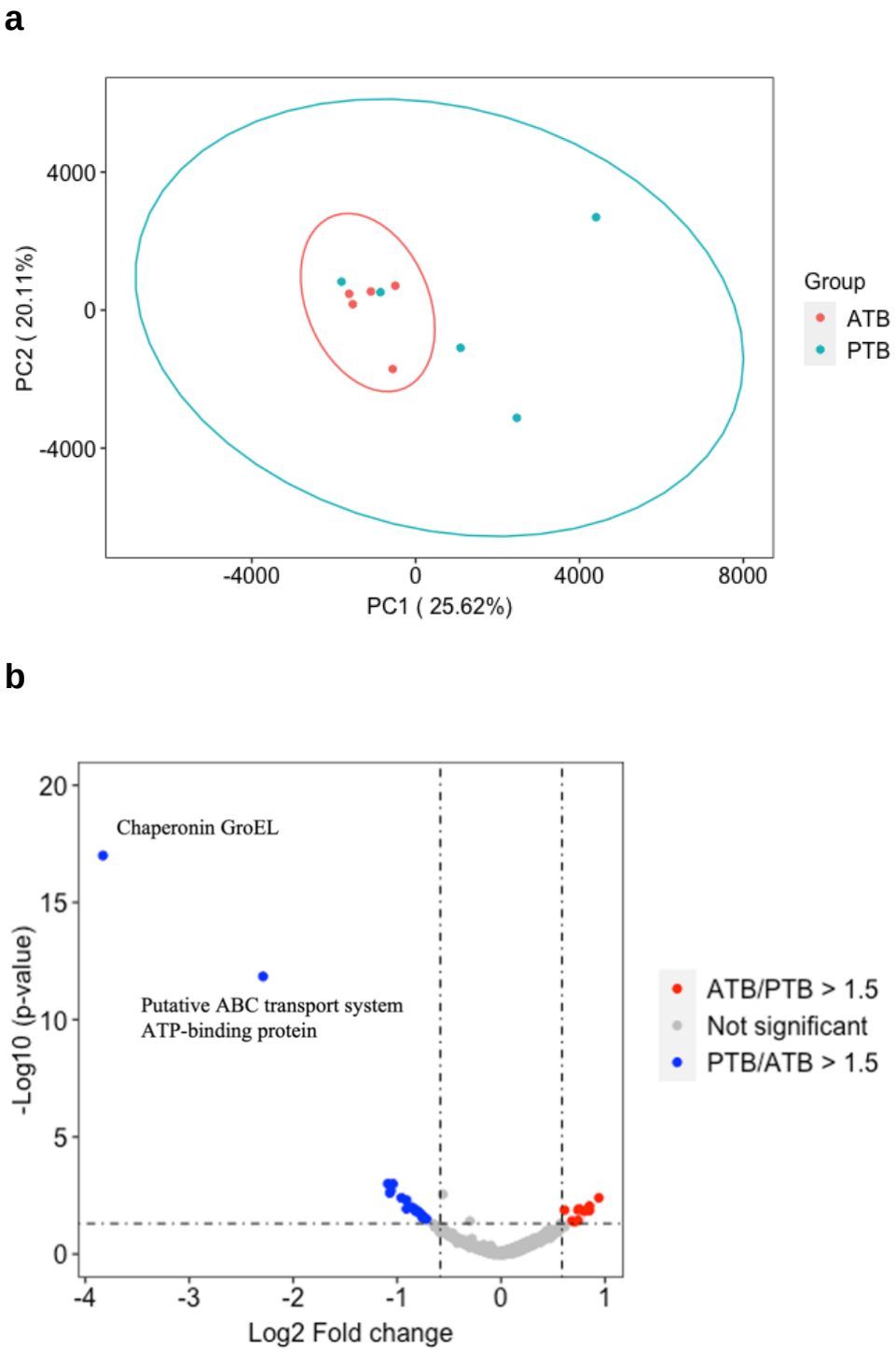


Figure 5-5. (a) The 20 most abundant archaeal proteins, from methanogenic genera, identified in the bovine faeces; and (b) the 20 biological processes in which most faecal archaeal peptides were involved.

5.3.1.4 Differentially represented proteins

The 10 faecal samples were plotted using their scores in principal component 1 (PC1: 25.62 %) and principal component 2 (PC2: 20.11 %) (Figure 5-6 a). No obvious clustering by diet was noted. Figure 5-b shows a volcano plot of all the identified bovine, barley and microbial proteins on the two diets. In total, there were 39 in 846 proteins differentially abundant ($p < 0.05$ and ATB/PTB > 1.5 or PTB/ATB > 1.5) between diets (Table 5-2), with more differential proteins being more abundant in the PTB group (28/39), and only 11 proteins in the ATB group were in higher abundance than those in PTB.

Sixteen host proteins were found to be differentially abundant in comparisons, among them, only four proteins were more abundant (1.6- to 1.9-fold) in the ATB group than the PTB group. Proteins including outer dense fibre protein 2 (3.5-fold), DNA helicase (2.1-fold), DAZ interacting zinc finger protein 1 (2.1-fold) and 2'-5'-oligoadenylate synthase-like protein (2.1-fold) were more abundant in the PTB group compared to the ATB group. Fifteen barley proteins were found to be differentially abundant in the two groups. Four of them were more abundant in ATB than those in PTB, including DNA mismatch repair protein (1.6-fold) and DIRP domain-containing protein (1.8-fold). Hexosyltransferase and IPPc domain-containing protein were 5.2-fold higher in the PTB group compared to those in the ATB group. Four bacterial proteins and four archaeal proteins were differentially abundant in the two groups. *Clostridium* chaperonin GroEL was found to be 14.2-fold higher in the PTB group compared to that in the ATB group. Putative ABC transport system ATP-binding protein in *Methanococcus* was 4.9-fold higher in the PTB group than that in ATB. Polysaccharide deacetylase family protein in *Methanobacterium* was 2.1-fold higher in PTB compared to that in ATB.



The two vertical dotted lines represent fold change of ATB and PTB equal to 1.5 or 2/3, and the horizontal dotted line represents *p*-value equals to 0.05. Only proteins between the two groups with a fold change > 1.5 or < 2/3 and $p < 0.05$ were considered differentially abundant proteins.

Figure 5-6. (a) Scatter plot of the PCA of the bovine faecal samples, and (b) volcano plot of all identified faecal bovine, barley and microbial proteins.

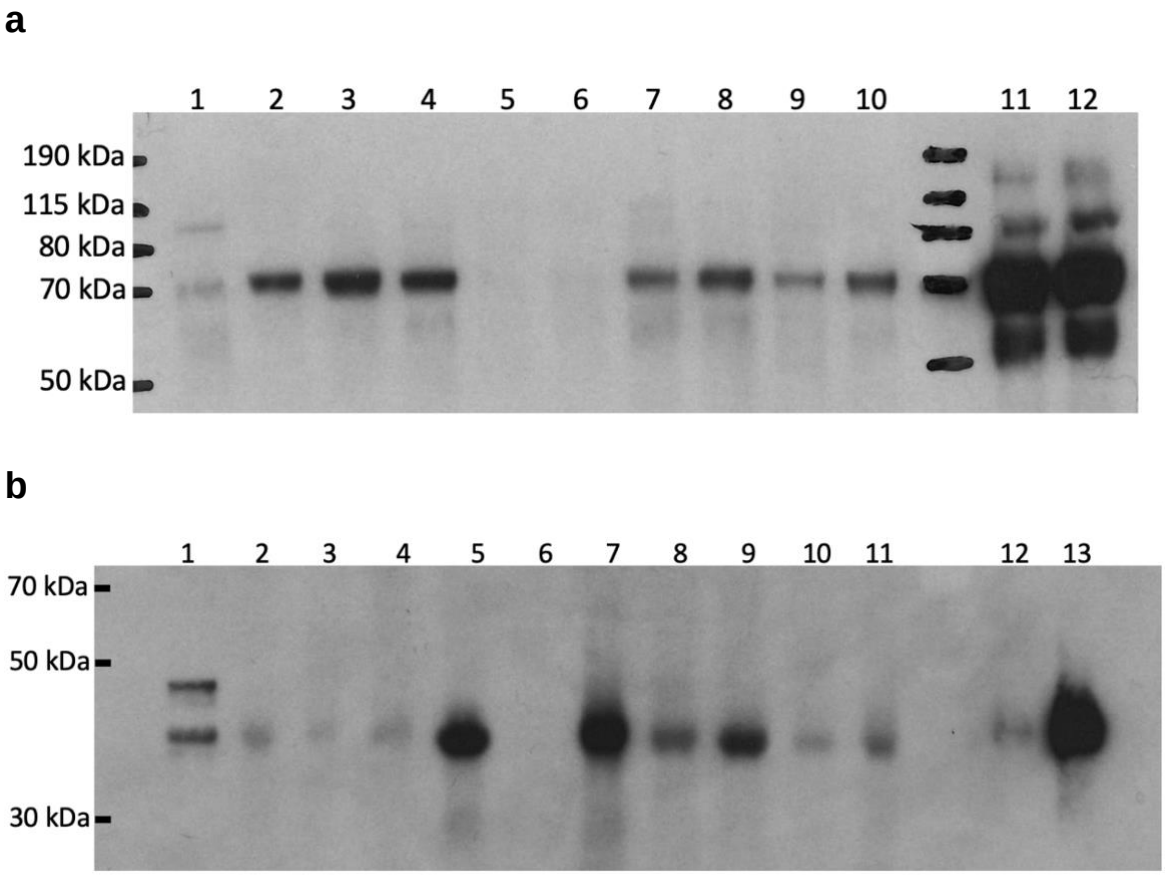
Table 5-2. Differentially abundant proteins in contrast between ATB and PTB groups.

No	Protein	Accession	Log2(ATB/PTB)	P - value	Adj. p
	Bovine				
1	Outer dense fibre protein 2	Q2T9U2	-1.8	0.000	0.000
2	2'-5'-oligoadenylate synthase-like	F1MXX7	-1.09	0.001	0.033
3	DAZ interacting zinc finger protein 1	A0A3Q1MSE5	-1.06	0.002	0.066
4	DNA helicase	A0A6B0RAZ5	-1.04	0.001	0.027
5	Pericentriolar material 1	A0A3Q1LX22	-0.96	0.004	0.127
6	Calcium/calmodulin dependent protein kinase IG	F1N2U4	-0.86	0.010	0.240
7	Uncharacterized protein	A0A6B0RQQ4	-0.82	0.015	0.320
8	Ig-like domain-containing protein	G5E513	-0.76	0.023	0.405
9	Uncharacterized protein	A5PK72	-0.74	0.028	0.464
10	Small nuclear RNA activating complex polypeptide 4	E1BCK9	-0.74	0.027	0.460
11	Rho guanine nucleotide exchange factor 10-like protein	Q29RM4	-0.72	0.032	0.504
12	IgG-FC binding protein	G3X6I0	-0.72	0.032	0.504
13	Chymotrypsin-like elastase family member 1	Q28153	0.68	0.039	0.557
14	Dimethylaniline monooxygenase [N-oxide-forming] 3	Q8HYJ9	0.83	0.012	0.266
15	Calcineurin binding protein 1	G3X746	0.85	0.009	0.220
16	Proline rich coiled-coil 2A	E1BAF6	0.94	0.004	0.127
	Barley				
17	Hexosyltransferase	A0A287R109	-2.38	0.000	0.000
18	IPPc domain-containing protein	M0WHX3	-2.38	0.000	0.000
19	Uncharacterized protein	A0A287M8V1	-2.36	0.000	0.000
20	Uncharacterized protein	A0A287L083	-1.45	0.000	0.001
21	Uncharacterized protein	M0Y5F8	-1.38	0.000	0.000
22	Uncharacterized protein	M0YF16	-0.91	0.005	0.108
23	Uncharacterized protein	M0WS69	-0.83	0.013	0.225
24	Receptor-like serine/threonine-protein kinase	A0A287H0K6	-0.83	0.012	0.225
25	DUF4042 domain-containing protein	A0A287WUI8	-0.8	0.015	0.253
26	Predicted protein	F2EK19	-0.78	0.018	0.284

27	DCD domain-containing protein	A0A287VUU3	-0.77	0.020	0.304
28	DNA mismatch repair protein	F2E4X9	0.71	0.041	0.528
29	Predicted protein	F2EKY2	0.74	0.037	0.490
30	Uncharacterized protein	A0A287DX28	0.8	0.015	0.253
31	DIRP domain-containing protein	A0A287Q620	0.85	0.014	0.240
	Bacteria				
	Clostridium				
32	Chaperonin GroEL	A0A6M0H6D9	-3.83	0.000	0.000
33	AAA domain-containing protein	A0A1M6LVJ1	-0.91	0.012	0.672
34	Sigma-70 family RNA polymerase sigma factor	A0A6M0YFZ9	0.74	0.013	0.672
	Bacteroides				
35	N-6 DNA methylase	A0A7J5P0W5	0.75	0.012	0.672
	Archaea				
	Methanococcus				
36	Putative ABC transport system ATP-binding protein	A0A8J7UUA2	-2.29	0.000	0.000
	Methanobacterium				
37	Polysaccharide deacetylase family protein (Fragment)	A0A6A8RJZ8	-1.07	0.002	0.106
	Methanosphaera				
38	Hydroxymethylglutaryl-CoA synthase	A0A328SP32	-0.76	0.027	0.493
	Methanosarcina				
39	Phosphoesterase	A0A0F8CAC4	0.61	0.013	0.351

5.3.2 Validation of proteomics

The presence of serum albumin and serpin Z4 in bovine faeces was verified by western blot (Figure 5-7, full image in Appendix Figure 5-1). The positive controls for albumin detection on the WB (purified bovine serum albumin) and for serpin Z4 (barly extract and beer protein) confirmed the cross reactivities of the respective antibodies. The western blot demonstrated that there was wide variation in the amount of serum albumin and serpin Z4 among samples. However, there were no significantly differences between diet groups (p = 0.69 and p = 0.15, respectively), consistent with the results of comparison based on the relative abundance detected by proteomics (p = 0.48 and p = 0.12, respectively) (Figure 5-8).



Faecal samples in both gels were loaded interspersed between groups (30 µg bovine protein for serum albumin and 50 µg for serpin Z4): (a) lanes 1,3,5,7 and 9 were samples from ATB group, lanes 2,4,6,8 and 10 were from PTB group, lanes 11 and 12 were bovine serum controls (1 µg); (b) lanes 2,4,6,8 and 10 were samples from ATB group, lanes 3,5,7,9 and 11 were from PTB group, lane 1 was a barley control (0.4 µg) and, lanes 12 (0.2 µg) and 13 (0.4 µg) were barley-based beer (Original Beset, Belhaven, UK).

Figure 5-7. Western blot of (a) bovine serum albumin and (b) barley serpin Z4.

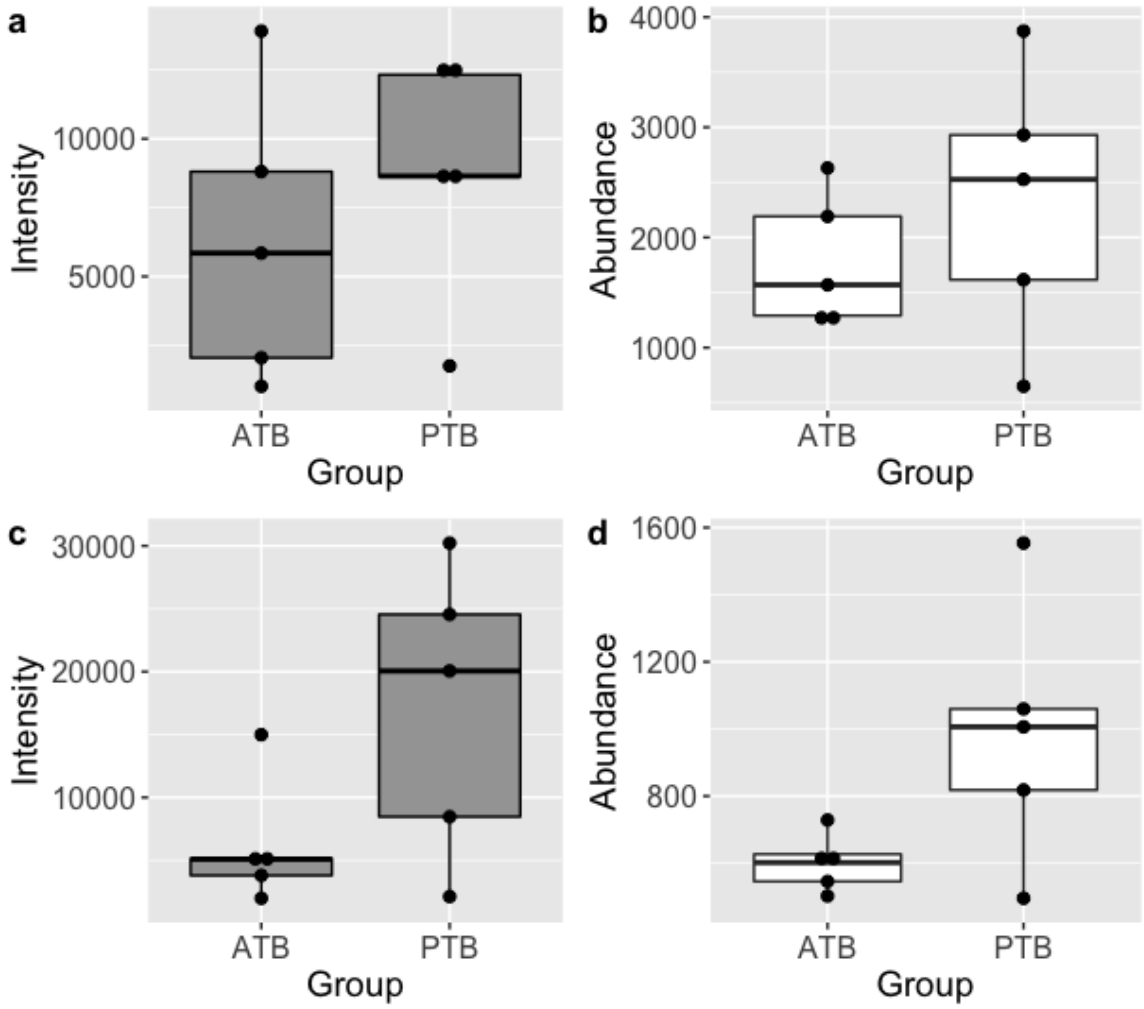


Figure 5-8. Albumin (a and b) and serpin Z4 (c and d) in the bovine faecal samples according to western blot (a and c) and proteomic analysis (b and d) determined by Image J analysis of the bands on western blot.

5.4 Discussion

The present study identified host, dietary and microbial proteins in bovine faeces. Bovine glycoproteins were abundant, and many digestive enzymes were detected, as well as a number of protease inhibitors. Protease inhibitors from barley, notably serpin Z4 and Z7 were also found in the samples, with serpin Z4 being the most abundant barley protein found in the faeces. Many microbial proteins were identified in the faecal samples. A large proportion of bacterial proteins were from *Clostridium*, and among the archaea, there were relatively abundant methanogenic proteins of *Methanobrevibacter*, *Methanosarcina* and *Methanobacterium*. The majority of differentially expressed proteins were more abundant in the faeces of animals on the PTB diet than on the ATB diet.

5.4.1 Bovine proteins identified in the cattle faeces

Faeces constantly sample the cellular environment when passing down the GIT: 281 host proteins were identified in the present study, including proteins derived from leakage, exfoliation and secretion. A relatively small number of host proteins was identified in the faeces compared to the examination of human faeces (834 proteins) (Bosch et al. 2017), likely because the bovine database was smaller than that of human. Relatively small numbers of host protein were also reported in faeces of mice (Oleksiewicz et al. 2005, Ang et al. 2010) and sheep (Palomba et al. 2018). Consistent with a previous study on sheep (Palomba et al. 2018), serum albumin, IgG-FC binding protein, mucin 2, cationic trypsin (serine protease 1), polymeric immunoglobulin receptor (PIGR) and mucosal pentraxin which were among the 20 most abundant proteins in sheep faeces, were also highly abundant in bovine faeces in the present study. Serum albumin in the GIT has been usually reported to be associated with protein-losing enteropathy (Levitt and Levitt 2016). Despite the absence of indicators of clinical disease, these animals were consuming high grain diets (14 kg DM/d of diets that were predominantly barley), and would be expected to have some degree of alteration to the gut as a consequence (Ferguson et al. 2022). Although high MW (> 200 KDa) and abundant glycoproteins were largely removed by using the IGSP method described previously (Huang et al. 2022), abundant glycoproteins such as mucin 2 and glycoprotein 2 were still detected in the present study. Mucin 2, mucin 5AC and mucin 13 identified in the faeces are secreted from goblet cells or Paneth cells, mucous cells and enterocytes, respectively. Mucins are highly glycosylated proteins, and besides providing lubrication, they are able to protect the GIT from bacteria and self-digestion by resisting endogenous proteases, especially the major intestinal mucin 2 (Johansson M.E.V

et al. 2013). Glycoprotein 2 is secreted by pancreas. It has been found to be co-localized with mucin 2, and is present in the outer mucin layer of the colon, playing an important role in defence against bacteria during intestinal inflammation (Kurashima et al. 2021). Among the proteins which were also involved in inflammation, besides PIGR which has been mentioned above, complement C3 and immunoglobulin J chain have also been identified in monkey faeces (Tsutaya et al. 2021). Mucosal pentraxin was abundant in the bovine faeces. It has been suggested to be a nutrient-sensitive biomarker of gut health because it is predominantly expressed in the healthy colonic mucosa of rats, strongly regulated by dietary heme and calcium (van der Meer-Van Kraaij et al. 2003, Drew et al. 2006, van der Meer-van Kraaij et al. 2007), and is involved in regulation of apoptosis, mediating the clearance of apoptotic epithelial cells as part of the normal cell turnover processes in healthy colonic mucosa or preventing apoptosis from diet-induced damage (van der Meer-Van Kraaij et al. 2003, van der Meer-van Kraaij et al. 2007). The observation of these proteins in the bovine faeces from proteomics may suggest a novel way to characterise the intestinal health of animals.

Many host enzymes related to intestinal digestion, were identified in the present study, including carboxypeptidase A1, cationic trypsin, phospholipase A2, membrane associated phospholipase A2 and pancreatic lipase-related protein 2 were among the top 20 most abundant host proteins. Trypsin and chymotrypsin are important endopeptidases secreted by the pancreas. Carboxypeptidase A1 is a zinc-dependent exopeptidase secreted from the pancreas and activated by trypsin in the duodenum (Auld 2013). It acts on smaller polypeptides after protein breakdown by endopeptidases, being important in the degradation of food proteins, and leading to the formation of amino acids (Auld 2013). Vendrell et al. (2000) proposed that carboxypeptidase A1 functioned only as a digestive enzyme. However, O'Reilly et al. (2021) found that carboxypeptidase A1 was highly abundant in faeces of the dogs with chronic bowel diseases compared to the healthy dogs, suggesting its potential as a biomarker of GI-related diseases in animals. Aminopeptidase N, mainly located in small-intestinal or renal microvillar membrane, was also found in the cattle faeces. It is important in the final digestion of peptides, and has been reported to be involved in cell motility and adhesion (Chen et al. 2012). Lysozyme detected in the bovine faeces stays active in ruminal acidic environment, functions as a digestive enzyme, and is antibacterial (Dominguez-Bello et al. 2004) and resistant to pepsin degradation (Dobson et al. 1984). Alpha-amylase and maltase-glucoamylase, which work synergistically in digesting starch in small intestine, were also identified in the samples. Although there was a significant difference in faecal starch concentration between groups (Chapter 3 Trial 1),

no differences were found in these enzymes; small sample sizes in the present study compared to the previous study might explain this difference.

Schmidt et al. (1995) demonstrated that proteins present in normal faeces of humans were mainly protease-resistant or locally secreted proteins, however, alpha-2-macroglobulin, a broad-spectrum protease inhibitor, was not detected in their study but was abundant in the present study. Besides inhibiting serine-, cysteine- and metalloproteinases, alpha-2-macroglobulin plays an important role in inflammation, immunity and infection (Ang et al. 2010, Park et al. 2021, Vandooren and Itoh 2021). High abundance of serpin A3-1 and serpin A3-7 were found in the bovine faeces. Serpin A3-1 selectively inhibited trypsin but not chymotrypsin, elastase or subtilisin (Hwang et al. 1999, Hook and Hwang 2005). Serpin A3-7 selectively inhibited papain-like cysteine and elastase-like serine proteases, but not chymotrypsin, trypsin, plasmin, thrombin, furin or cathepsin B (Hwang et al. 2002, Hook and Hwang 2005). Other proteins in the serpin family such as alpha-1-antiproteinase and serpin B1 were also identified in the bovine faeces. These findings, along with barley serpins, which will be discussed later, showed a very high concentration of protease inhibitors in bovine faeces and may reflect their high concentrations in the barley-based diet and resistance to digestion.

5.4.2 Barley serpin Z4 and Z7 in the cattle faeces

Barley serpin Z4 and serpin Z7 were identified in the cattle faeces, and the former was found to be the most abundant barley-origin protein in the faeces. The presence of serpin Z4 was confirmed by western blot, although the higher molecular band, probably serpin Z7, was not found in either the faeces or the barley-based beer, which requires further investigation. Most of the current research on serpin Z4 and serpin Z7 is related to beer production. They survive through malting and brewing in the beer making process (Picariello et al. 2012). Serpin Z4 was found to be positively correlated with beer foam and suggested to be a marker of foam stability (Evans et al. 1999), while correlation between serpin Z7 and beer foam was negative (Iimure and Sato 2013). The presence of such proteins from the diets might also have implications for foam formation in frothy bloat of cattle (Clarke and Reid 1974, Cheng et al. 1998) and would be worthy of future research to address this observation.

The protease inhibition of serpins and some other plant-derived protease inhibitors in the intestinal tract have been reported (Yoo et al. 2000, Alvarez-Alfageme et al. 2011, Karlund

et al. 2021) but their impact on digestion of the host has not been fully explained. This effect might be anti-nutritional since it may affect the digestion of food by competitive binding, inhibiting the action of digestive enzymes on proteins and leading to accumulation of undigested proteins or over-secretion of the digestive enzymes (Muzquiz et al. 2012, Karlund et al. 2021). Serpins can also be beneficial against pathogens or pests. For example, *Arabidopsis* AtSerpin 1 inhibited proteases from insects which rely on serine or cysteine proteases for digestion, thus to reducing the availability of amino acids for their growth and development (Alvarez-Alfageme et al. 2011); serpin-1 in pumpkin was negatively correlated with aphid survival, suggesting a potential role for plant serpins as insect inhibitors (Yoo et al. 2000). However, there is no study to date investigating serpin Z4 or Z7 in inhibiting animal digestive enzymes. If barley serpin Z4 and Z7 impair digestion by inhibiting proteases, knocking out the genes or inhibiting their expression in barley might be expected to enhance the absorption of nutrients in animals. Finally, some plant-derived serine protease inhibitors have been reported to play an important role in inflammatory responses in the GIT, mitigating inflammation and gastric pain (Karlund et al. 2021), and have anticarcinogenic properties (Srikanth and Chen 2016). The detection of serpin-Z4 and serpin-Z7 in the cattle faeces may provide a new research direction for ruminant digestion and absorption, and diseases.

5.4.3 Microbial proteins in the bovine faeces

In the present study, bacterial proteins from *Clostridium* were predominant, followed by *Prevotella*, *Bacteroides*, *Ruminococcus* and *Eubacterium*. Dowd et al. (2008) and Callaway et al. (2010) also found that *Clostridium* was the most abundant bacterial genera in bovine faeces, and reported the same gene enrichment order of the five genera as our findings except that the order of *Prevotella* and *Bacteroides* was reversed. The most common archaea in ruminants are methanogens (Janssen and Kirs 2008). Most methane from ruminants is emitted by eructation, but the flatus that is resulted from fermentation in hindgut (Murray et al. 1976) can also contribute to approximately 10 % of methane emission (Hill et al. 2016, Bekele et al. 2022). Our findings were consistent with previous studies, identifying many proteins of methanogenic archaea in the faeces. Among them, *Methanobrevibacter* was the dominant genus, followed by *Methanosarcina* and *Methanobacterium*. Previous studies reported that genes of *Methanobrevibacter* were commonly found in the gut of ruminants and *Methanobrevibacter* was regarded as the most representative archaea in ruminants (Miller 2002, St-Pierre and Wright 2013). Faeces share many microbial communities with the rumen. Archaeal genera *Methanobrevibacter*

(100%) and *Methanosphaera* (99.2%) were present in nearly all the ruminal and faecal samples of cattle from 52 genomic studies (Holman and Gzyl 2019). Using 16S rRNA sequencing and PCR-denaturing gradient gel electrophoresis, Zhou et al. (2014) found that phylotypes close to *Methanobrevibacter* were the main taxon in the GIT of calves. St-Pierre and Wright (2013) also reported that 16s rRNA gene sequences of *Methanobrevibacter* were the most frequently identified phylotypes in herbivores' guts. Genera *Prevotella* and *Ruminococcus* were considered core bacteria as they were present in most studied bovine faeces (Durso et al. 2010, Kim et al. 2014) as well as rumen samples (Henderson et al. 2015, Holman and Gzyl 2019). *Clostridium* and *Bacteroides* were abundant in the rectum (Mao et al. 2015, Zaheer et al. 2017), and they were enriched in the cecum and colon of calves compared to the rumen and were increased in the cecum as calf grew (Dias et al. 2018). Variations in composition and abundance of faecal microbial community exist among animal individuals (Bergmann 2017, Zaheer et al. 2017, Noel et al. 2019). Researchers have proposed that, in general, differences in rumen microbial community compositions are mainly attributable to diets (de Menezes et al. 2011, Cersosimo et al. 2016), with the host being less influential (Henderson et al. 2015). The differences in faecal microbial structure between the present and other studies might be partly explained by different dietary compositions, feeding operations, age, breed and geographical location of the host (Hook et al. 2010, Shanks et al. 2011, Kim et al. 2014, Henderson et al. 2015).

In the present study, faecal bacterial proteins were mainly involved in carbohydrate metabolism; glycolytic process; gluconeogenesis; glycose metabolism; cellular amino acid metabolic process and translation. Archaeal proteins were mainly involved in carbohydrate metabolism; translation; regulation of transcription, DNA-templated; and DNA repair, replication and recombination. Our findings were consistent with a multi-omics study on sheep faeces, which also demonstrated that the sheep faecal microbiota was primarily involved in catabolism (Tanca et al. 2017). Durso et al. (2011) and Mao et al. (2015) found that a high proportion of the faecal bacterial genes was related to carbohydrate and protein metabolism. The biological processes mentioned above had been predicted to be present throughout the GIT of cattle by metagenomic studies. Bacterial metabolism can produce short-chain fatty acids from undigested dietary plant polysaccharides in the GIT, contributing significantly to host energy balance (Flint et al. 2008, Precup and Vodnar 2019). Starch and sucrose metabolism has been found to be a core pathway of rumen bacteria, as is the metabolism of hydrolytic products such as glucose, maltose and xylose (Wang et al. 2013, Li and Guan 2017). Many enzymes in carbohydrate metabolism were

identified in the faeces in the present study, and similar results were reported in a sheep faecal proteomics study, showing glycolysis and peptidoglycan biosynthesis were the three most abundant potential metabolic pathways (Tanca et al. 2017). Methane from ruminants accounts for a large portion of greenhouse gas (Chang et al. 2019, Ugbogu et al. 2019, Bekele et al. 2022). It can be synthesized by methanogens using H_2, CO_2, formate, methyl compounds or acetate as substrates (de la Fuente et al. 2019). Models have been developed to predict the methane emission from dietary input variables such as dry matter intake, digestibility of hemicellulose, and metabolizable energy intake (Kebreab et al. 2008, Ramin and Huhtanen 2013). Some researchers found that animals which had lower residual feed intake could produce less methane (Hegarty et al. 2007). Many dietary strategies, including using secondary plant metabolites (e.g., tannins and saponins) (Ku-Vera et al. 2020), seaweed and 3-nitrooxypropanol (Almeida et al. 2021), have been proposed for methane mitigation in ruminants. The identification of archaeal proteins in the faeces may lead to more accurate prediction of methane emissions from the ruminants, and may also provide new ideas for reducing methane emissions.

5.4.4 Differentially abundant proteins in the bovine faeces

Most of the differentially abundant proteins with statistical significance were more abundant in PTB than those in ATB. Starch degradation was suggested to be specific for *Bacteroidetes* and was one of the most relevant metabolic pathways of sheep faecal microbiome (Tanca et al. 2017). In our previous study, the faecal starch concentration was lower in ATB than PTB. However, identified proteins related to starch utilization system such as TonB-dependent receptors (Tanca et al. 2017) from *Bacteroidetes* and *Prevotella*, were not found to differ between groups. The diversity of bacterial community was significantly decreased as digesta passed through the GIT (Frey et al. 2010), and the diet-induced changes in the rumen bacteria were reduced or eliminated in the faeces (Noel et al. 2019), which might explain the identifications of few differentially abundant proteins between groups. The adaptation of animals to the diets, the small sample size, the large variation between animals, and the integrity of the database also contributed to the results. Further studies are needed to have a deeper understanding of the treatment of diet on faeces, but the identification of the proteins here could provide a foundation.

5.5 Conclusion

The present study characterised a bovine faecal proteome of 281 bovine, 199 barley, 176 bacterial and 190 archaeal proteins. Mucin 2 was the most abundant host protein identified in the faeces, and many host digestive enzymes and protease inhibitors were also found. Barley serpin Z4 was the most abundant barley protein identified in the faeces, and serpin Z7 was also present. Among microbial proteins, a large proportion of bacteria were *Clostridium*, and *Methanobrevibacter* was the dominant archaeal genus. The majority of differentially abundant proteins between groups were more abundant in the PTB group compared to the ATB group.

5.6 Author contribution

Except that the samples were from Trial 1 of Chapter 3, collected by Gui Wolff and Nicholas Jonsson, I was responsible for the lab work, data analyses, visualisation and thesis writing.

Chapter 6 General Discussion and Conclusions

6.1 General discussion

The growth of cattle directly or indirectly reflects their health status and the effectiveness of feeding and management on farm, and the growth performance of cattle is directly related to economic return. Accurate estimation of cattle growth is potentially helpful, not only for monitoring animal welfare and on-farm diet management, but also for research into disease treatments and the development of new zootechnical products. The original overall aim of the study was to develop models for cattle that could efficiently use high-density bodyweight (BW) data for analysing instantaneous changes in BW and feed efficiency, thus enabling real-time characterisation of animal health status and prediction of growth potential. The work described in Chapter 2 was originally intended to form a platform for this work. However, due to constraints on accessing the facilities (financial and COVID-19), the direction of the project was changed. After determining the most suitable models for cattle, the effects of the ammonia treatment of cereal grains on animal growth performance and faecal proteome were studied, which was expected to lay a foundation for future studies on faecal indicators of growth efficiency. No power analysis was undertaken in Chapter 2 because the sample size we aimed for was the maximum possible size on the farms that were investigated. In relation to chapter 4 and 5, a power analysis was not needed because they were pilot studies and were not really testing a specific hypothesis.

Analysis of animal growth performance is usually retrospective and has been mostly based on infrequent observations of BW. However, as seen from the high-density BW data in Chapter 2, sigmoid trajectories better describe growth over the whole lifetimes of cattle, and more suitable growth models for different life stages were generated: the von Bertalanffy model for the whole of life growth of the lactating Holstein-Friesian cows; the exponential model for the calves up to four months old; the linear model and the negative exponential model for finishing beef cattle for the last 100 days or so before slaughter.

As an important indicator of performance and efficiency, growth rate has been widely used to evaluate the effects of interventions such as dietary changes (Soberon et al. 2012), diseases and treatments (Windeyer et al. 2014). The traditional method of quantifying growth rate is based on before-and-after measurement, which produces a linear slope

(ADG). However, the growth trajectories of animals are generally non-linear. Some researchers have split growth periods of cattle into shorter periods, calculated the growth rate of each period by using the traditional method and then obtained their mean values as the final estimates to minimize the error (Donovan et al. 1998, Malhado et al. 2013). Having found the most suitable models of the animal growth at different life stages based on the high-density BW records, accurate growth rate estimations were realized. Application of the exponential model improved the precision of parameter estimation and the sensitivity of analysis of known correlates of growth of calves up to four months old compared with the traditional linear model.

Automated monitoring of BWs is increasingly common on commercial farms. The units used in the present study were Biocontrol units for calves (Agri-EPI Centre Ltd, UK) and Beef Monitor crates for finishing cattle (Ritchie Agricultural, UK), which automatically weighed animals when they drank milk replacer or water. The lactating cows were weighed on exit from the parlour, using a walk-over scales and a shedder to ensure an accurate weight for each individual cow. Other automatic animal weighing systems such as LiveStock Planner (Hencol AB, Sweden) (Segerkvist et al. 2020), GrowSafe Systems (BioAlberta, Canada) and automatic walk-over scales (Gargiulo et al. 2018, González-García et al. 2018) have also been used in practice, both indoors and outdoors. Precision livestock farming, which aims to monitor animals by near-continuous real-time information, including BWs, water and feed intake, and animal behaviour, has been advocated for many years. The model-based control has been suggested to be a key component of precision livestock farming systems (Berckmans 2017). By using these automatic weighing systems, the near-continuous BW data has been proposed to provide early-warning for abnormal weight gains of on-pasture cattle, caused by parasitic disease (Segerkvist et al. 2020); to show small short-term changes in lactating dairy cows (Dickinson et al. 2013); and to be used to check daily physiological status of dairy cows in the first 100 days in milk and to provide suggestions about dietary management (Alawneh et al. 2011).

In Chapter 2, there were challenges in analysing the data directly extracted from the equipment. For example, animal movement lead to inaccurate measurement of BWs, and the position where the animal stood (partial animal or more than one animal on the scales) was also important. By using linear regression to fit splines to the raw data or using a MCMC method, removing the outliers and calculating the mean value of multiple daily BWs, the data were able to be analysed. Many issues have also been reported in studies

(Segerkvist et al. 2020), in addition to improvements to equipment, including incorporating sensors that can track and record livestock activity to improve the accuracy of BW measurement, baits are needed to attract animals to the weighing equipment. This is particularly challenging for animals on pasture, where the food or water provided at the equipment is not the only source. Prior to analysis, some researchers removed the extreme values at the whole group level and outliers which were out of the expected daily BW range (based on actual static BW) at individual level, respectively (González-García et al. 2018), or generated nonparametric cubic spline regression model to remove potential errors and calculated the mean daily measurement (Alawneh et al. 2011).

According to the results of modelling growth of the young fattening beef cattle, there was little benefit in using non-linear models, which was confirmed in the study of effects of the enzyme-catalysed ammonia treatment of cereal grains on production performance of young fattening beef cattle in Chapter 3. Multiple BWs of each young fattening beef animal were collected during the feeding period and the growth models were fitted. The linear model was found to fit relatively better than the non-linear models. One of the reasons was that the time for slaughter was generally decided based on farmers' experience on evaluation of animal fat and body condition score, so that most animals that were removed for slaughter were likely to be still in the almost linear phase of the logistic curve and not close to the expected mature BWs. This was supported by the findings in Chapter 2 that the longer the finisher cattle stayed on the farm, the more likely the best model was to be the negative exponential model. On the basis of the work described in this chapter, we propose that the application of an exponential model to calf growth should result in improved accuracy and power to detect the effects of experimental interventions.

Ammonia treatment has been considered to be a suitable method for the preservation of cereal grains that are harvested with a high moisture content. The ammonia treatment can not only inhibit contamination caused by microorganisms, but also enhance nutritional value and might promote nutrient utilization in animals, thereby improving animal production performance. Most of the previous studies that demonstrated beneficial effects of ammonia treatment on the growth performance of beef cattle used direct insufflation of bagged cereal grains with anhydrous ammonia gas (Laksesvela 1981, Goonewardene et al. 1998). Although many advantages of ammonia treatment of animal feed have been reported (Robinson and Kennelly 1989, Belanche et al. 2021), there are contradictory findings and some issues. For example, in some studies, no improvements in dry matter intake, average daily gain (ADG), feed efficiency or carcass traits were found in animals

that were fed ammonia-treated diets (Mowat et al. 1981, Yaremcio et al. 1991, Bradshaw et al. 1996). There were palatability problems with the ammonia treatment which might have resulted in decreased feed intake, but could be solved by exposing the feed to air prior to feeding (Mandell et al. 1988). The ammonia treatment of the diet increased water intake of animals, which might require more bedding if there was increased urine production (Ørskov 1979). Direct insufflation with anhydrous ammonia usually required specific equipment and was costly and laborious. The enzyme-catalysed ammonia treatment is a commercially available method of cereal grain preservation using enzymic catalysis of the conversion of urea to ammonia. Grain is mixed with urea and a source of urease, together with sufficient water to allow the reaction to occur, then deposited in commodity bays and covered with plastic sheeting for 7-10 days, during which ammonia gas percolates through the cereal grains and is absorbed by the grain. In Chapter 3, this method of ammonia treatment showed similar effects to those previously reported in studies using direct insufflation with anhydrous ammonia, improving the growth performance of cattle in beef fattening systems, and addressing laborious and time-consuming problem.

Consistent with those previously reported in studies using direct insufflation with anhydrous ammonia, the enzyme-catalysed ammonia treatment of grain decreased feed conversion ratio (FCR) of cattle. The improvements might be because of increased digestibility of the cereal grains and increased nutrient utilisation attributed to the increased microbial growth arising from nitrogen. Cattle fed the enzyme-catalysed ammonia-treated diets had lower faecal starch concentrations, which was consistent with either increased digestibility of the grain or increased utilisation. There was inconsistency among studies in the results of the volatile fitty acid (VFA) analysis, which may be due in part to the timing of sampling in relation to dietary changes and timing of the last meal. Cattle on a higher rate of starch supplementation were likely to adapt by developing a higher rate of VFA clearance, which in turn would result in lower concentrations of VFAs if sampling was carried out post-mortem and several hours after the last meal (Jonsson et al. 2019).

Our study was intended to contrast the performance of cattle fed on an ammonia-treated cereal diet in comparison with a locally more traditional diet, and was mainly focused on contrasting the effect of the feeding system rather than any independent, direct effect of the ammonia treatment (diets were broadly equivalent in energy and protein). Preservation of cereal grains is essential in Scotland, while it is not required in Italy, thus the comparisons in the two commercial feeding systems were different. In Scotland, the barley-based diet was treated with either enzyme-catalysed ammonia or propionate; in Italy, the maize-based

diet was with or without enzyme-catalysed ammonia treatment. A treatment with urea to catalyse its conversion to ammonia was not used in this study because it is not an accepted method of preservation and because some (possibly most) of the urea would be expected to be converted to ammonia as a result of endogenous cereal enzymes (Patra and Aschenbach 2018), reducing the validity of the control treatment. Fewer animals in the Italian study that were fed the enzyme-catalysed ammonia-treated diets developed bloat or had higher faecal scores, which were considered to be indicative of diarrhoea, than those fed the untreated diet or the propionate-treated diet. However, the disease incidence was low overall and not tested statistically. Trial 1 was conducted on a traditional Scottish fattening unit, with limited capacity for subdivision, meaning that only two pens were allocated per treatment. Although the effects of pen on the analyses was tested and found not to be significant, analysis with pen as the unit of observation nonetheless indicated that there might be a significant effect of treatment on the FCR.

The overarching aim of our work on faecal proteomics is to identify indicators (biomarkers) of growth (metabolism) efficiency, health, or the suitability of nutritional inputs in the faeces of cattle, little research has been done on protein in ruminant faeces, especially on proteins from host animals or on proteins from the feed. Due to the complex composition of faeces, a clean-up process prior to the proteomics might be necessary to remove substances which affect the downstream analysis. Therefore, we conducted a preliminary study (Chapter 4) to develop a workflow to first clean up the faecal samples and then detect and identify host proteins, as well as proteins of plant and microbial origin in the cattle faeces. We conducted the study with archived faecal samples of the cattle that were fed ammonia-treated and propionate-treated barley-based diet (Trial 1 in Chapter 3) to identify the abundant bovine, dietary and microbial proteins in faeces, and to contrast the proteomes of the cattle fed the two diets.

A relatively small number of protein identifications were obtained in the initial pilot study, in which sample preparation was based on filter-aided sample preparation (FASP) method. Faecal proteins in SDS-PAGE gels were stained by periodic acid-Schiff (PAS), and abundant high molecular weight (MW) glycoproteins were thought to have detrimental effects on the sample preparation for proteomic analysis, possibly causing the low yield of identified proteins. This was confirmed by optimising sample preparation using the method (in-gel sample preparation, IGSP) we developed in Chapter 4, which removed the high MW glycoproteins and increased the number of protein identifications. In this method, we ran samples for a short-distance in the polyacrylamide gel and excised the specific areas of

the protein staining bands prior to in-gel digestion, which avoided the influence of MS-incompatible detergents, buffers or salts, and removed the abundant high MW glycoproteins. There were still some limitations of IGSP application, for example, a small number of proteins that might have been partially digested to peptides would be retained in samples prepared by FASP, while they would be removed in IGSP; and proteins outside the selected MW range (10 - 190 kDa) could be lost; manual excising of specific areas of the gels could introduce errors; and the influence of gel pieces on digestion efficiency and peptide recovery should also be noted. Although we were aware of these limitations, the effect of IGSP for the greater number of protein identification was likely to outweighed them.

A mixture of host, dietary and microbial proteins in the cattle faeces was revealed by nanoflow ultrahigh-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (nUHPLC-ESI-MS/MS) with tandem mass tag (TMT) quantification, and was validated by western blotting of bovine serum albumin and barley serpin Z4, as described in Chapter 5. A bolus of faeces effectively samples its environment as it passes through the gastrointestinal tract - host proteins derived from leakage, exfoliation and secretion were detected, including proteins that were involved in inflammation, proteins that have been found to be abundant in the intestine including enzymes and protease inhibitors. There were not as many host proteins as reported in human faecal proteomes (Debyser et al. 2016, Bosch et al. 2017). The smaller database of cattle compared to those of human might be one of the reasons. Although abundant high MW glycoproteins were largely removed by using IGSP, some glycoproteins were still abundant in the faeces, suggesting important roles in the cattle gut. Investigation of these host proteins in the faeces might suggest a way to characterise the intestinal health status of animals. Among dietary proteins, barley serpin Z4 and serpin Z7 were identified in the cattle faeces, with serpin Z4 being the most abundant barley-origin protein. To our knowledge, this was the first time that these barley proteins have been found in animal faeces. Serpin Z4 and serpin Z7 have been mostly studied in relation to beer-brewing due to their involvement in the stability of foam in beer (Evans et al. 1999, Iimure et al. 2011, Iimure and Sato 2013). They can survive malting and brewing (Iimure et al. 2010, Picariello et al. 2012). The interaction of serpin Z4 and serpin Z7 with host proteins, especially digestive enzymes, is something that our group will study in the future, and the dietary proteins identified in the faeces are expected to provide information about animal digestion. Because the databases we used contained those from Swissprot and TrEMBL, in which TrEMBL is a computer-

annotated sequence database, many of the barley proteins and bacterial proteins that were identified require further annotation.

Many microbial proteins were identified in the faeces as well, with *Clostridium* as the predominant bacterial genus in the cattle faeces, followed by *Prevotella*, *Bacteroides*, *Ruminococcus* and *Eubacterium*; and for archaea, there were relatively abundant methanogenic proteins of *Methanobrevibacter*, *Methanosarcina* and *Methanobacterium*. These findings were consistent with some genomic studies (Dowd et al. 2008, Callaway et al. 2010), but there were also differences in faecal microbial structure between the present and further studies, which should be due to the differences relating to the platform and databases, the differing predispositions of the approaches to assign a peptide or sequence, differences between genetic potential and actual protein expression, and might also be explained by different dietary compositions and feeding operations (Shanks et al. 2011, Kim et al. 2014). The major biological processes in which the microbial proteins were over-represented showed their important roles in carbohydrate and protein metabolism.

Most proteins did not differ significantly between groups. This might be because faecal samples were collected more than two months after switching to these diets, by which time the cattle had already adapted to these diets. In general, there was a high level of inter-animal, within-group variation. Only 5 genera of the bacteria and 30 archaeal methanogenic genera were searched in the study because larger databases would result in higher computational load and increased false discovery rates. There are highly conserved sequences among microbial species of the same genus, which increases the difficulty of identifying microbiota to species. Many tools for metaproteomics analysis such as MetaProteomeAnalyzer can help classify bacteria to genera levels, however, TMT-quantified proteins could not be easily identified. In the present study, microbial proteins with at least two peptides matched were included in analyses, some proteins might be screened out, but the results still provided a foundation for future studies. Protein expression is constantly changing in space and time. Our results reflected the faecal proteomes of these cattle in this specific condition, and were expected to inform the development of our future studies to optimize diets for cattle and potential modify performance metrics.

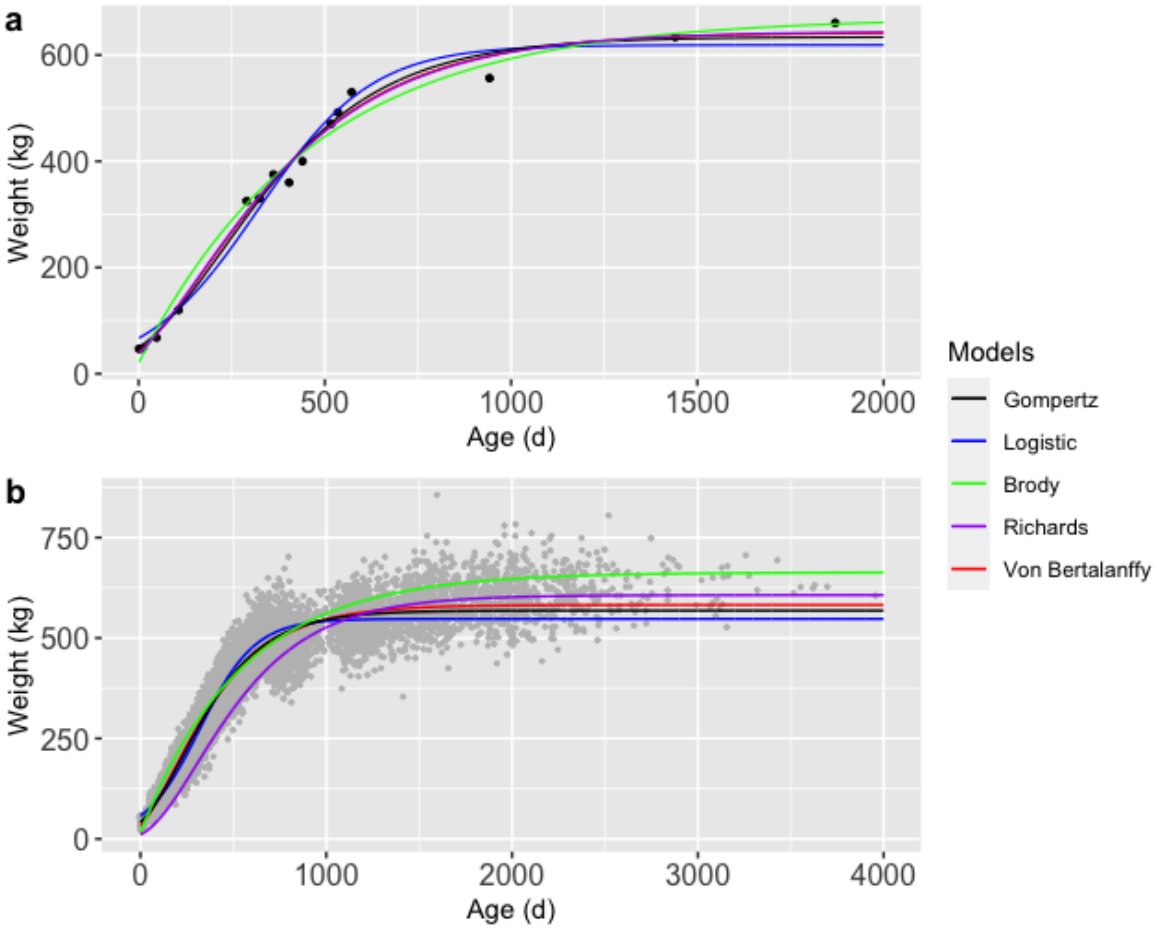
For the next step of our studies, we are going to predict the sites of production of the most abundant host proteins identified in this thesis, with possibly functional implications as well, based on the published bovine gene expression atlas and single-cell RNA databases.

Next, to characterise faecal proteomes in more animals (30 sheep and 130 cattle), firstly while at pasture, then contrast these with the same groups of animals after transition to a typical commercial fattening diet. Finally, parallel examinations of proteomes and metagenomes are expected to be conducted on previously collected faecal samples to determine whether a phylogenetic or functional protein group strategy is likely to be preferable for biomarker discovery.

6.2 Conclusions

First of all, the study established a workflow of determining the best model for growth of cattle at different life stages by using high-density bodyweight data. Application of the most suitable model improved the accuracy of parameter estimation and analysis of intervention effects on growth of calves, potentially providing greater statistical power in analyses, using the same number of animals. Next, the enzyme-catalysed ammonia treatment of cereal grains had similar effects to those previously reported in studies using direct insufflation with anhydrous ammonia, improving the production performance of cattle in beef fattening systems, possibly by increasing nutrient utilization. Then, an in-gel sample preparation method was developed to clean up the faecal protein samples prior to LC-MS/MS and improved faecal protein identifications. Finally, although the effects of enzyme-catalysed ammonia treatment on faecal proteome remain to be further studied, the bovine faecal proteome composed of host, dietary, and microbial proteins was revealed. These studies will provide a foundation for future studies of bovine GI-related diseases and optimizing diets for cattle to improve performance and efficiency.

Appendix

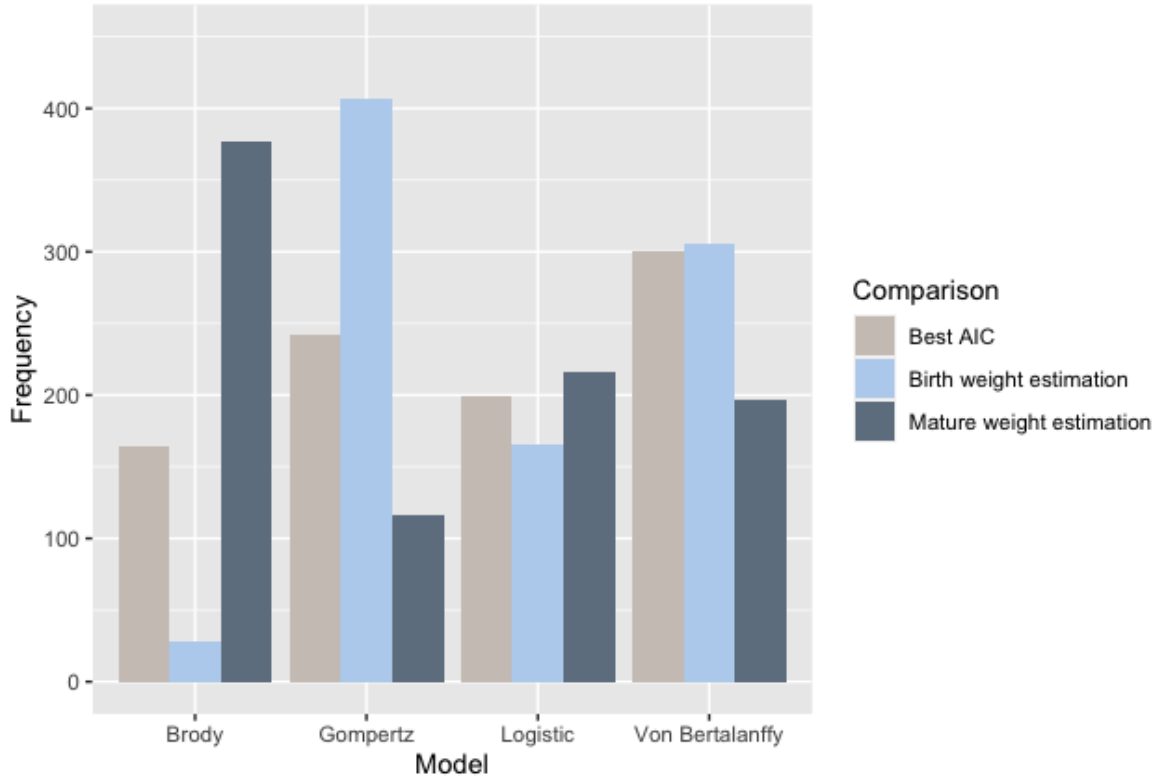


Appendix Figure 2-1. The whole of life bodyweights (BW) of cattle in the LBW data set (the BWs during lactations was taken the lightest daily mean BW for that lactation) and the predicted growth trajectories of models: (a) an example of fitting models to an individual cattle (black dots are the BWs and the coloured lines are the predicted growth trajectories of models); (b) the grey dots are all the BWs from 906 cattle in this data set, and the coloured curves are the predicted growth trajectories of models, of which the parameters were the mean values of all the cattle.

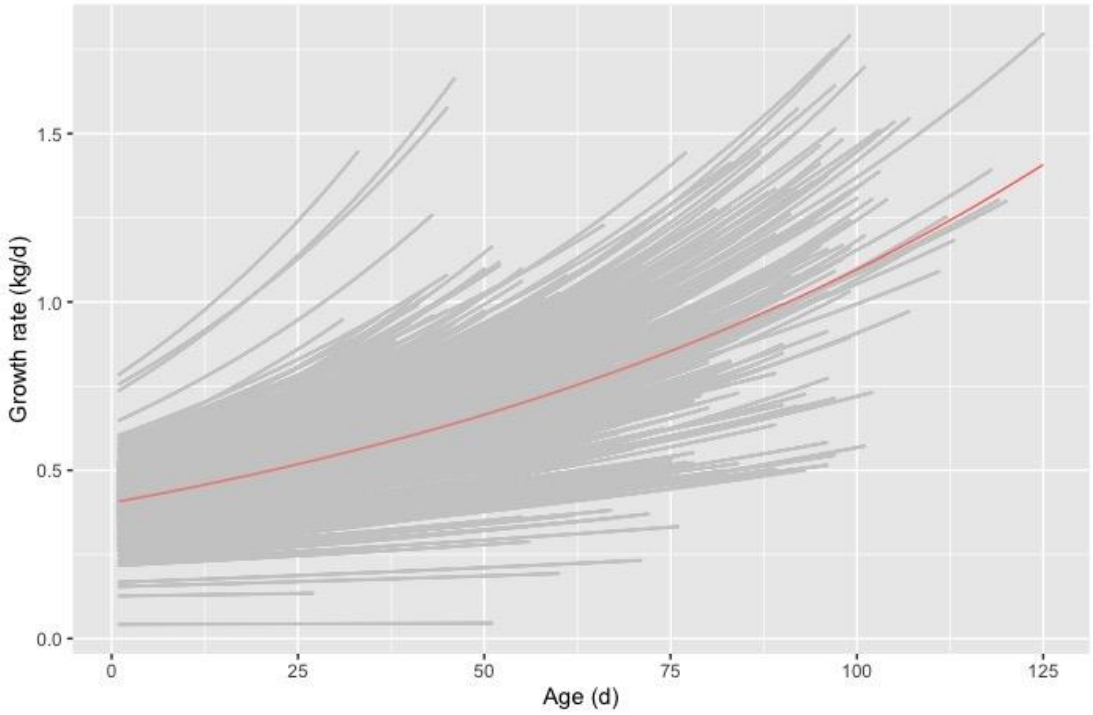
Appendix Table 2-1. The results of fitting models to BWs of 906 cows from birth to 4,000 days old in the LBW data set, in which the BWs during lactations was taken the lightest daily mean BW for that lactation. Results are presented as mean (standard deviation) unless indicated otherwise.

Estimate		Growth model				
		Logistic	**Brody**	**Gompertz**	**Von Bertalanffy**	**Richards**
Number of successful models fitted		906/906	905/906	906/906	906/906	523/906
R^2		0.9828 (0.0116)	0.9798 (0.0153)	0.9869 (0.0105)	0.9868 (0.0111)	0.9919 (0.0074)
AIC		142.31 (35.85)	143.85 (34.20)	136.95 (35.33)	136.67 (34.77)	135.35 (34.04)
RMSE		26.64 (8.49)	22.89 (8.52)	18.44 (7.87)	18.32 (7.83)	15.57 (6.64)
Parameter *A*		547.46 (56.56)	663.31 (165.34)	568.21 (59.07)	581.76 (70.94)	606.57 (74.53)
Parameter *B*		8.58 (2.03)	0.98 (0.02)	2.62 (0.31)	0.61 (0.06)	0.70 (0.24)
Parameter *k*		0.0067 (0.0011)	0.0018 (0.0004)	0.0042 (0.0007)	0.0034 (0.0006)	0.0028 (0.0009)
Estimated birthweight (kg)		63.09 (16.00)	-3.23 (30.25)	44.18 (14.71)	33.92 (15.23)	29.17 (10.63)
Birthweight difference (estimated-observed)	RMSE	25.98	54.36	14.41	17.00	16.40
	Mean difference (SD)	+20.82 (15.55)	-45.50 (29.77)	+1.90 (14.29)	-8.35 (14.81)	-13.11 (9.87)
Mature BW difference (estimated-observed)	RMSE	40.56	142.97	39.05	53.38	99.47
	Mean difference (SD)	-29.39 (27.96)	+62.88 (128.47)	-10.42 (37.66)	+1.94 (53.37)	+18.31 (97.87)

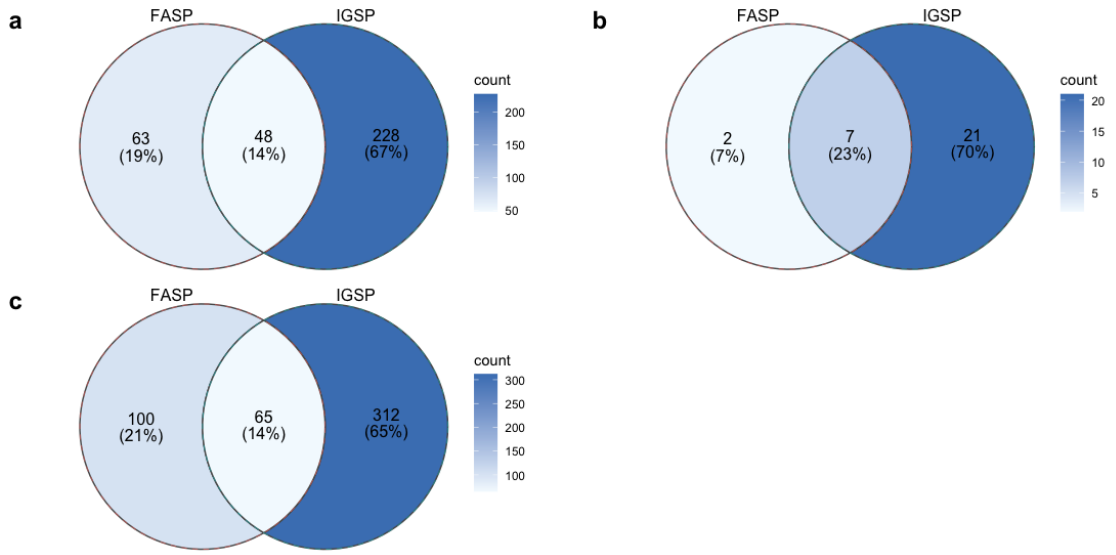
Parameter *A*, *B* and *k* are parameters in the models, represent mature BW, integration parameter and maturation rate, respectively. RMSE of birthweight or mature BW: square root of the mean of the differences between estimated birthweights/ mature BWs of models and the corresponding observations of all the animals. Mean (SD) of birthweight or mature BW estimation: mean value and standard deviation of the differences between the estimated birthweights/ mature BWs and the actual observations of all the animals, '+' means over-estimated, '-' means under-estimated.



Appendix Figure 2-2. Model comparison according to goodness-of-fit, and estimations of birthweight and mature BW for the individual cow in the LBW data set, in which the BWs during lactations was taken the lightest daily mean BW for that lactation. Each column represents the number of cows for which the model provides the best fit.



Appendix Figure 2-3. The growth rate trajectories predicted by the exponential model for all the 361 calves (grey lines) from day of birth until their last day on measurement and for the herd (red line) from 1 to 125 days old.



Appendix Figure 4-1. Comparisons between filter-aided sample preparation (FASP) and in-gel sample preparation (IGSP) in the bovine faecal sample identifications: (a) bovine proteins; (b) barley proteins and (c) bacterial proteins (*Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae*, *Prevotellaceae*, *Bacterioidaceae*, *Spirochaetaceae*). Proteins compared here were all identified master proteins.

Appendix Table 4-1. The full list of proteins with at least two peptides identified by filter-aided sample preparation (FASP) and in-gel sample preparation (IGSP) in the bovine faecal samples (50 *vs* 284).

No	Protein	Peptide coverage (%)	Peptides	PSMs	Unique peptides	MW (kDa)	Sequest HT score
	IGSP						
1	P02769 Albumin [OS=Bos taurus]	57	29	145	29	69.2	453.83
2	Q7SIH1 Alpha-2-macroglobulin [OS=Bos taurus]	26	28	81	28	167.5	236.7
3	P15396 Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 [OS=Bos taurus]	30	16	70	16	99.5	227.96
4	P81265 Polymeric immunoglobulin receptor [OS=Bos taurus]	28	13	37	13	82.4	120.3
5	Q2UVX4 Complement C3 [OS=Bos taurus]	9	12	37	12	187.1	96.34
6	P13214 Annexin A4 [OS=Bos taurus]	34	9	35	9	35.9	109.2
7	P19111 Intestinal-type alkaline phosphatase [OS=Bos taurus]	28	9	34	9	57.1	96.74
8	P24627 Lactotransferrin [OS=Bos taurus]	18	9	18	9	78	57.52
9	P81425 Dipeptidyl peptidase 4 [OS=Bos taurus]	15	9	13	9	88.3	40.68
10	Q3T166 Mucosal pentraxin [OS=Bos taurus]	49	8	29	8	24.7	96.83

11	P34955 Alpha-1-antiproteinase [OS=Bos taurus]	25	8	26	8	46.1	71.65
12	P79098 Aminopeptidase N [OS=Bos taurus]	10	8	21	8	109.2	59.61
13	Q9TTE1 Serpin A3-1 [OS=Bos taurus]	23	8	30	3	46.2	83.69
14	Q8SPP7 Peptidoglycan recognition protein 1 [OS=Bos taurus]	41	6	19	6	21.1	61.59
15	Q3SZM7 Dipeptidase 1 [OS=Bos taurus]	20	6	16	6	45.1	50.09
16	Q1JPB0 Leukocyte elastase inhibitor [OS=Bos taurus]	16	6	13	6	42.2	39.09
17	P17453 Bactericidal permeability-increasing protein [OS=Bos taurus]	13	6	9	6	53.4	22.26
18	Q06284 Lysozyme C-3 [OS=Bos taurus]	61	6	25	3	16.3	82.62
19	A2I7N0 Serpin A3-4 [OS=Bos taurus]	16	6	22	1	46.3	59.34
20	P00760 Cationic trypsin [OS=Bos taurus]	30	5	23	5	25.8	79.65
21	P00732 Carboxypeptidase B [OS=Bos taurus]	20	5	13	5	47.3	35.28
22	Q06285 Lysozyme C-1 [OS=Bos taurus]	51	5	22	2	16.3	73.4
23	Q28153 Chymotrypsin-like elastase family member 1 [OS=Bos taurus]	27	4	20	4	28.5	59.1
24	P23132 Lithostathine [OS=Bos taurus]	33	4	14	4	19.3	45.19
25	P28783 Protein S100-A9 [OS=Bos taurus]	41	4	14	4	17.1	40.6

26	P46193 Annexin A1 [OS=Bos taurus]	15	4	10	4	38.9	29.08
27	Q95323 Carbonic anhydrase 4 [OS=Bos taurus]	15	4	10	4	35.1	25.15
28	Q7M3E1 Chymotrypsin-C [OS=Bos taurus]	21	4	8	4	29.2	24.83
29	Q58CQ9 Pantetheinase [OS=Bos taurus]	13	4	6	4	56.9	17.69
30	A2I7N3 Serpin A3-7 [OS=Bos taurus]	10	4	7	4	46.9	15.96
31	P06394 Keratin, type I cytoskeletal 10 [OS=Bos taurus]	6	4	6	4	54.8	15.17
32	P13753 BOLA class I histocompatibility antigen, alpha chain BL3-7 [OS=Bos taurus]	15	4	10	3	41.5	30.06
33	P00730 Carboxypeptidase A1 [OS=Bos taurus]	9	3	11	3	47.1	29.95
34	P41361 Antithrombin-III [OS=Bos taurus]	8	3	10	3	52.3	29.03
35	Q29461 Chymotrypsin-like elastase family member 2A [OS=Bos taurus]	15	3	8	3	28.8	27.46
36	A1L595 Keratin, type I cytoskeletal 17 [OS=Bos taurus]	5	3	6	3	48.7	17.11
37	P04272 Annexin A2 [OS=Bos taurus]	12	3	6	3	38.6	16.37
38	Q9TTK8 Creatine kinase U-type, mitochondrial [OS=Bos taurus]	9	3	4	3	46.9	11.07

39	Q9N2I2 Plasma serine protease inhibitor [OS=Bos taurus]	11	3	4	3	45.3	10.56
40	P00766 Chymotrypsinogen A [OS=Bos taurus]	12	2	8	2	25.7	26.32
41	Q29463 Anionic trypsin [OS=Bos taurus]	14	2	8	2	26.3	24.87
42	A6QNM2 Ribosome-releasing factor 2, mitochondrial [OS=Bos taurus]	4	2	7	2	86	24.64
43	O46415 Ferritin light chain [OS=Bos taurus]	18	2	7	2	20	22.94
44	Q56JZ2 Phospholipase A2, membrane associated [OS=Bos taurus]	17	2	6	2	16.3	18.47
45	P00829 ATP synthase subunit beta, mitochondrial [OS=Bos taurus]	5	2	6	2	56.2	18.1
46	Q28050 Protein S100-A7 [OS=Bos taurus]	34	2	6	2	11.5	13.93
47	P05805 Proproteinase E [OS=Bos taurus]	8	2	6	2	27.3	13.87
48	P79345 NPC intracellular cholesterol transporter 2 [OS=Bos taurus]	18	2	5	2	16.6	13.84
49	Q6R8F2 Cadherin-1 [OS=Bos taurus]	3	2	4	2	97.9	12.73
50	Q3T0Z2 Gastrotropin [OS=Bos taurus]	36	2	3	2	14.5	9.18
51	Q5EA79 Galactose mutarotase [OS=Bos taurus]	9	2	3	2	37.6	8.14

52	Q2YDP6 Testis-expressed protein 35 [OS=Bos taurus]	10	2	3	2	24.5	6.97
53	P60712 Actin, cytoplasmic 1 [OS=Bos taurus]	6	2	3	2	41.7	6.95
54	O46382 Brefeldin A-inhibited guanine nucleotide-exchange protein 1 [OS=Bos taurus]	2	2	3	2	208.6	6.41
55	P62285 Abnormal spindle-like microcephaly-associated protein homolog [OS=Bos taurus]	1	2	5	2	395.6	6.27
56	F1N4M2 Myelin regulatory factor-like protein [OS=Bos taurus]	5	2	2	2	100	5.23
57	P28782 Protein S100-A8 [OS=Bos taurus]	30	2	2	2	10.5	5.14
58	Q27960 Sodium-dependent phosphate transport protein 2B [OS=Bos taurus]	3	2	2	2	75.8	4.05
59	Q5XQN5 Keratin, type II cytoskeletal 5 [OS=Bos taurus]	3	2	6	1	62.9	19.01
60	P13752 BOLA class I histocompatibility antigen, alpha chain BL3-6 [OS=Bos taurus]	6	2	5	1	40.3	13.91
61	Q148H7 Keratin, type II cytoskeletal 79 [OS=Bos taurus]	4	2	4	1	57.7	11.73
62	A2I7M9 Serpin A3-2 [OS=Bos taurus]	23	8	30	3	46.2	83.69
63	Q3ZEJ6 Serpin A3-3 [OS=Bos taurus]	16	6	22	1	46.3	59.34

64	P08728 Keratin, type I cytoskeletal 19 [OS=Bos taurus]	5	3	6	3	43.9	17.11
65	P63258 Actin, cytoplasmic 2 [OS=Bos taurus]	6	2	3	2	41.8	6.95
66	Q29S21 Keratin, type II cytoskeletal 7 [OS=Bos taurus]	4	2	6	1	51.5	19.01
67	Q08D91 Keratin, type II cytoskeletal 75 [OS=Bos taurus]	3	2	6	1	59	19.01
68	Q06283 Lysozyme C-2 [OS=Bos taurus]	61	6	25	0	16.3	82.62
69	A2I7N1 Serpin A3-5 [OS=Bos taurus]	12	5	19	1	46.4	49.25
70	P04421 Lysozyme C [OS=Bos taurus]	46	5	17	0	16.4	49.49
71	A2I7N2 Serpin A3-6 [OS=Bos taurus]	9	4	16	0	46.4	41.77
72	P05785 Keratin, type I cytoskeletal 14 [OS=Bos taurus]	12	2	4	2	10.7	11.03
73	P06293 Serpin-Z4 [OS=Hordeum vulgare]	25	7	27	7	43.2	85.9
74	P11643 Alpha-amylase/trypsin inhibitor CMd [OS=Hordeum vulgare]	40	4	11	4	18.5	34.04
75	Q43492 Serpin-Z7 [OS=Hordeum vulgare]	11	4	11	3	42.8	28.71
76	P32936 Alpha-amylase/trypsin inhibitor CMb [OS=Hordeum vulgare]	28	3	4	3	16.5	10.03
77	P13691 Alpha-amylase inhibitor BDAI-1	30	3	5	3	16.4	14.79

	[OS=Hordeum vulgare]						
78	P42210 Phytepsin [OS=Hordeum vulgare]	7	3	6	3	54.2	17.52
79	P49970 Signal recognition particle 54 kDa protein 3 [OS=Hordeum vulgare]	6	2	2	2	53.8	4.51
80	P16968 Alpha-amylase inhibitor BMAI-1 [OS=Hordeum vulgare]	17	2	5	2	15.8	18.02
81	Q40066 Serpin-ZX [OS=Hordeum vulgare]	6	2	4	1	42.9	9.41
82	P24295 NAD-specific glutamate dehydrogenase [OS=Clostridium symbiosum]	32	11	61	11	49.3	189.26
83	C4Z0Q6 Phosphoenolpyruvate carboxykinase (ATP) [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]	31	10	71	5	59	208.23
84	A9KRZ3 Elongation factor G [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	14	9	30	6	78.1	90.62
85	C4ZBL1 Phosphoenolpyruvate carboxykinase (ATP) [OS=Agathobacter	24	9	77	2	59	227.31

	rectalis (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)]						
86	C4ZB99 Elongation factor Tu [OS=Agathobacter rectalis (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)]	26	7	37	3	43.4	112.56
87	P22983 Pyruvate, phosphate dikinase [OS=Clostridium symbiosum]	9	6	11	6	96.6	28.92
88	B2UYT8 Ketol-acid reductoisomerase (NADP(+)) [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	22	6	34	5	37	101.54
89	C4Z2R9 Elongation factor Tu [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]	20	6	30	3	44	90.83
90	Q8A463 Elongation factor Tu [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	16	5	42	4	43.6	132.75
91	Q0TMN0 Elongation factor Tu [OS=Clostridium perfringens (strain ATCC 13124 / DSM	19	5	40	3	43.5	129.06

	756 / JCM 1290 / NCIMB 6125 / NCTC 8237 / Type A)]						
92	Q8A414 Phosphoenolpyruvate carboxykinase (ATP) [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	11	5	32	2	59.1	96.17
93	Q9RQ13 L-fucose isomerase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	9	5	9	1	65.7	22.65
94	Q64MV4 Phosphoenolpyruvate carboxykinase (ATP) [OS=Bacteroides fragilis (strain YCH46)]	10	5	27	1	59	86.78
95	Q5LIN8 L-fucose isomerase [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	11	5	11	1	65.3	32.02
96	Q8A4N6 Polyribonucleotide nucleotidyltransferase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC	7	4	7	4	78.3	17.3

	10582 / E50 / VPI-5482)]						
97	B2TIG8 DNA-directed RNA polymerase subunit beta [OS=Clostridium botulinum (strain Eklund 17B / Type B)]	4	4	7	4	140.6	16.69
98	G3KIM4 Lactoyl-CoA dehydratase subunit alpha [OS=Anaerotignum propionicum]	10	4	15	4	47.4	40.03
99	P94316 NAD-specific glutamate dehydrogenase [OS=Bacteroides fragilis (strain YCH46)]	11	4	12	4	48.4	31.57
100	A9KSJ1 60 kDa chaperonin [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	9	4	10	3	57	24.74
101	P95544 NAD(P)-specific glutamate dehydrogenase [OS=Prevotella ruminicola]	12	4	17	3	48.8	47.84
102	C4ZD46 60 kDa chaperonin [OS=Agathobacter rectalis (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)]	9	4	5	3	57.1	14.07
103	Q64NK6 Elongation factor G	7	4	10	3	77.5	30.7

	[OS=Bacteroides fragilis (strain YCH46)]						
104	C4Z3R4 60 kDa chaperonin [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]	8	4	9	2	57.4	27.47
105	Q890N8 Elongation factor G [OS=Clostridium tetani (strain Massachusetts / E88)]	6	4	13	2	77.1	36.25
106	B2TIX0 60 kDa chaperonin [OS=Clostridium botulinum (strain Eklund 17B / Type B)]	11	4	6	2	57.9	15.9
107	A6TWI9 DNA-directed RNA polymerase subunit beta' [OS=Alkaliphilus metalliredigens (strain QYMF)]	4	4	11	1	130.4	28.72
108	A6TWI5 Elongation factor G [OS=Alkaliphilus metalliredigens (strain QYMF)]	7	4	12	1	76	33.65
109	B1IFD4 60 kDa chaperonin [OS=Clostridium botulinum (strain Okra / Type B1)]	11	4	6	1	57.9	14.55
110	A6L1X1 Adenylosuccinate synthetase [OS=Bacteroides	5	3	5	3	46.8	14.91

	vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]						
111	Q07064 Formate--tetrahydrofolate ligase [OS=Clostridium cylindrosporum]	9	3	7	3	59.1	16.29
112	Q97EX9 Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B 2 [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	6	3	3	3	54.6	6.32
113	B9DYW1 Formate--tetrahydrofolate ligase [OS=Clostridium kluyveri (strain NBRC 12016)]	6	3	4	3	60.4	9.51
114	Q64PM7 Glucose-6-phosphate isomerase [OS=Bacteroides fragilis (strain YCH46)]	7	3	9	3	48.7	22.06
115	Q8A490 30S ribosomal protein S8 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	27	3	10	3	14.6	25.77
116	Q0SRE3 Chaperone protein DnaK	5	3	4	3	66.5	9.41

	[OS=Clostridium perfringens (strain SM101 / Type A)]						
117	Q8A479 50S ribosomal protein L2 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	15	3	7	3	29.8	19.78
118	A6KXA0 60 kDa chaperonin [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	4	3	5	3	58.1	11.97
119	Q8A1G1 TonB-dependent receptor SusC [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	3	3	7	3	111.1	16.06
120	C4Z2V6 30S ribosomal protein S4 [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]	16	3	6	3	22.7	13.82
121	Q59309 Glyceraldehyde-3-phosphate dehydrogenase [OS=Clostridium pasteurianum]	6	3	15	3	36.1	36.12

122	A0PXN0 Formate--tetrahydrofolate ligase [OS=Clostridium novyi (strain NT)]	8	3	8	2	60.1	30.55
123	B9DYB5 30S ribosomal protein S3 [OS=Clostridium kluyveri (strain NBRC 12016)]	19	3	6	2	24.7	17.08
124	Q5LD89 ATP synthase subunit beta [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	8	3	7	1	55.2	20.74
125	Q890N5 DNA-directed RNA polymerase subunit beta' [OS=Clostridium tetani (strain Massachusetts / E88)]	3	3	7	1	126.9	18.47
126	A5N5D7 60 kDa chaperonin [OS=Clostridium kluyveri (strain ATCC 8527 / DSM 555 / NCIMB 10680)]	9	3	4	1	58.1	11.54
127	A9KJL3 DNA-directed RNA polymerase subunit beta' [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	2	3	8	1	140.8	20.43
128	A9KK92 ATP synthase subunit beta	8	3	7	1	51.5	21.19

	[OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]						
129	Q8KJ24 60 kDa chaperonin [OS=Clostridium botulinum]	10	3	4	0	58.2	10.51
130	A6LPX8 Ketol-acid reductoisomerase (NADP(+)) [OS=Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)]	7	2	3	2	36.6	6.37
131	Q64NL4 30S ribosomal protein S3 [OS=Bacteroides fragilis (strain YCH46)]	7	2	3	2	27.1	6.68
132	A6LPI6 Asparagine--tRNA ligase [OS=Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)]	4	2	4	2	53.3	9.46
133	A6KYH0 DNA-directed RNA polymerase subunit alpha [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	6	2	7	2	37.5	20.02
134	C4Z2R7 30S ribosomal protein S7 [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 /	17	2	2	2	17.7	6.51

	VPI C15-48 / C15-B4)]						
135	A6L0V1 30S ribosomal protein S2 [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	8	2	4	2	30.5	10.52
136	G3KIM3 Lactoyl-CoA dehydratase subunit beta [OS=Anaerotignum propionicum]	7	2	9	2	41.8	27.08
137	A9KNV3 Acetate kinase [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	7	2	7	2	42.6	15.44
138	Q8A4A1 30S ribosomal protein S4 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	8	2	5	2	22.6	11.57
139	A6KYH2 30S ribosomal protein S11 [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	18	2	4	2	13.9	9.14
140	Q97GU6 Aspartate--tRNA ligase [OS=Clostridium acetobutylicum	5	2	2	2	68.2	4.54

	(strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]						
141	A6TVN9 ATP-dependent helicase/deoxyribonuclease subunit B [OS=Alkaliphilus metalliredigens (strain QYMF)]	3	2	2	2	134.4	4.45
142	Q8KNX9 Enolase [OS=Bacteroides fragilis (strain YCH46)]	7	2	2	2	46.4	5.19
143	A9KJI8 30S ribosomal protein S3 [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	14	2	3	2	24.2	7.39
144	A6LPL4 Transcription elongation factor GreA [OS=Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)]	19	2	2	2	17.8	4.13
145	C4ZBS3 30S ribosomal protein S19 [OS=Agathobacter rectalis (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)]	24	2	7	2	10.3	19.1
146	Q97LM4 Maltose-6'-phosphate glucosidase MalH [OS=Clostridium	6	2	3	2	49.9	6.57

	acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]						
147	C1FLJ7 N-acetylmuramic acid 6-phosphate etherase [OS=Clostridium botulinum (strain Kyoto / Type A2)]	11	2	3	2	32.6	6.71
148	A0PXU1 30S ribosomal protein S12 [OS=Clostridium novyi (strain NT)]	19	2	3	2	13.8	6.55
149	A0Q151 Alanine--tRNA ligase [OS=Clostridium novyi (strain NT)]	3	2	2	2	98.6	4.23
150	Q5L9B6 30S ribosomal protein S18 [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	13	2	4	2	10.7	11.35
151	A9KJI2 50S ribosomal protein L5 [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	11	2	2	2	20.2	4.33
152	Q8A476 50S ribosomal protein L3 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	11	2	5	2	21.9	14.15

153	A6TWF3 DNA-directed RNA polymerase subunit alpha [OS=Alkaliphilus metalliredigens (strain QYMF)]	6	2	6	2	34.8	14.84
154	A0Q0E9 Ketol-acid reductoisomerase (NADP(+)) [OS=Clostridium novyi (strain NT)]	10	2	2	2	36.7	4.55
155	A5N2N7 Triosephosphate isomerase [OS=Clostridium kluyveri (strain ATCC 8527 / DSM 555 / NCIMB 10680)]	11	2	3	2	27.3	7.2
156	A6KYR3 Methionine--tRNA ligase [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	4	2	3	2	77.7	6.58
157	B9E1I8 30S ribosomal protein S2 [OS=Clostridium kluyveri (strain NBRC 12016)]	12	2	8	1	26.5	20.98
158	A0Q2T1 60 kDa chaperonin [OS=Clostridium novyi (strain NT)]	7	2	4	1	58.1	11.98
159	C4ZBS5 30S ribosomal protein S3 [OS=Agathobacter rectalis (strain ATCC	15	2	6	1	24	17.8

	33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)]						
160	Q97EI4 30S ribosomal protein S3 [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	13	2	5	1	24.7	14.99
161	Q64PM9 Lysine--tRNA ligase [OS=Bacteroides fragilis (strain YCH46)]	5	2	3	1	66.2	9.33
162	Q8XKU0 Phosphoglycerate kinase [OS=Clostridium perfringens (strain 13 / Type A)]	6	2	3	1	42.7	8.83
163	A6TWH2 50S ribosomal protein L14 [OS=Alkaliphilus metalliredigens (strain QYMF)]	19	2	2	1	13.4	4.54
164	A9KQ65 Ketol-acid reductoisomerase (NADP(+)) [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	8	2	8	1	36.7	28.27
165	C4ZBS9 50S ribosomal protein L14 [OS=Agathobacter rectalis (strain ATCC 33656 / DSM 3377 /	19	2	2	1	13.3	4.55

	JCM 17463 / KCTC 5835 / VPI 0990)]						
166	B2UY23 Phosphoglycerate kinase [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	6	2	2	1	42.4	4.76
167	A9KNC4 30S ribosomal protein S2 [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	8	2	11	1	27.6	28.6
168	A8MLC4 Elongation factor Tu [OS=Alkaliphilus oremlandii (strain OhILAs)]	8	2	27	1	43.9	80.74
169	C1FND0 Formate--tetrahydrofolate ligase [OS=Clostridium botulinum (strain Kyoto / Type A2)]	5	2	4	1	60.7	12.71
170	Q8A5W4 Lysine--tRNA ligase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	4	2	4	1	65.9	11.83
171	Q0TMP3 Elongation factor G [OS=Clostridium perfringens (strain ATCC 13124 / DSM 756 / JCM 1290 / NCIMB 6125 /	3	2	6	1	76	15.33

	NCTC 8237 / Type A)]						
172	P94598 Glutamate dehydrogenase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	5	2	10	1	49	23.63
173	Q97JG4 Glycerol kinase [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	5	2	6	1	55.6	12.87
174	A6LPQ8 Elongation factor G [OS=Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)]	3	2	5	1	75.8	12.66
175	A6TWI4 Elongation factor Tu 1 [OS=Alkaliphilus metalliredigens (strain QYMF)]	8	2	27	1	43.7	90.4
176	B2V358 Glycerol kinase [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	5	2	2	1	54.8	4.49
177	A6KYK9 Elongation factor Tu [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 /	16	5	42	4	43.5	132.75

	NBRC 14291 / NCTC 11154)]						
178	P33165 Elongation factor Tu [OS=Bacteroides fragilis (strain YCH46)]	16	5	42	4	43.6	132.75
179	Q64ZS4 L-fucose isomerase [OS=Bacteroides fragilis (strain YCH46)]	11	5	11	1	65.3	32.02
180	B2UYA3 DNA-directed RNA polymerase subunit beta [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	4	4	7	4	139.5	16.69
181	Q5L7Z7 Polyribonucleotide nucleotidyltransferase [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	7	4	7	4	78.3	17.3
182	Q8A474 Elongation factor G [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	7	4	10	3	77.5	30.7
183	B2UZ02 60 kDa chaperonin [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	11	4	6	2	57.8	15.9

184	A6KYI1 30S ribosomal protein S8 [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	27	3	10	3	14.6	25.77
185	Q5L8C3 30S ribosomal protein S8 [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	27	3	10	3	14.6	25.77
186	P26823 Chaperone protein DnaK [OS=Clostridium perfringens (strain 13 / Type A)]	5	3	4	3	66.4	9.41
187	B2TI01 Asparagine--tRNA ligase [OS=Clostridium botulinum (strain Eklund 17B / Type B)]	4	2	4	2	53.3	9.46
188	Q8A5S6 30S ribosomal protein S18 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	13	2	4	2	10.7	11.35
189	Q8A3M1 Methionine--tRNA ligase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC	4	2	3	2	77.6	6.58

	10582 / E50 / VPI-5482)]						
190	A9KJJ0 30S ribosomal protein S19 [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	24	2	7	2	10.5	19.1
191	Q64NK8 50S ribosomal protein L3 [OS=Bacteroides fragilis (strain YCH46)]	11	2	5	2	21.8	14.15
192	Q8A482 30S ribosomal protein S3 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	7	2	3	2	27.1	6.68
193	Q5L7I8 Methionine--tRNA ligase [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	4	2	3	2	77.6	6.58
194	A6KYI9 30S ribosomal protein S3 [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	7	2	3	2	27.1	6.68
195	C4Z2T4 30S ribosomal protein S19 [OS=Lachnospira	24	2	7	2	10.3	19.1

	eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]						
196	Q64MP7 Methionine--tRNA ligase [OS=Bacteroides fragilis (strain YCH46)]	4	2	3	2	77.5	6.58
197	B2UXS0 Asparagine--tRNA ligase [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	4	2	4	2	53.3	9.46
198	A6KWD6 30S ribosomal protein S18 [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	13	2	4	2	10.6	11.35
199	Q0STD5 Phosphoglycerate kinase [OS=Clostridium perfringens (strain SM101 / Type A)]	6	2	3	1	42.7	8.83
200	A6TWJ8 Elongation factor Tu 2 [OS=Alkaliphilus metalliredigens (strain QYMF)]	8	2	27	1	43.7	90.4
201	C3KVU4 Formate--tetrahydrofolate ligase [OS=Clostridium botulinum (strain 657 / Type Ba4)]	5	2	4	1	60.7	12.71

202	A7FZB0 Formate--tetrahydrofolate ligase [OS=Clostridium botulinum (strain ATCC 19397 / Type A)]	5	2	4	1	60.7	12.71
203	Q5L9E5 Lysine--tRNA ligase [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	5	2	3	1	66.2	9.33
204	C4Z2U0 50S ribosomal protein L14 [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]	19	2	2	1	13.3	4.55
205	B2TPX4 Phosphoglycerate kinase [OS=Clostridium botulinum (strain Eklund 17B / Type B)]	6	2	2	1	42.5	4.76
206	A8MHG9 30S ribosomal protein S2 [OS=Alkaliphilus oremlandii (strain OhILAs)]	9	2	11	1	26.4	28.6
207	A0Q0S0 30S ribosomal protein S2 [OS=Clostridium novyi (strain NT)]	9	2	11	1	26.3	28.6
208	B1IGY1 Formate--tetrahydrofolate ligase [OS=Clostridium	5	2	4	1	60.7	12.71

	botulinum (strain Okra / Type B1)]						
209	Q8XHS1 Elongation factor G [OS=Clostridium perfringens (strain 13 / Type A)]	3	2	6	1	75.9	15.33
210	A6TRM3 30S ribosomal protein S2 [OS=Alkaliphilus metalliredigens (strain QYMF)]	9	2	11	1	26.5	28.6
211	A9KRZ4 Elongation factor Tu [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	16	5	35	0	43.8	114.9
212	A0PXU3 Elongation factor G [OS=Clostridium novyi (strain NT)]	5	3	11	0	76.1	32
213	P30717 60 kDa chaperonin [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	9	3	4	0	58	11.07
214	A8MLD7 Elongation factor G [OS=Alkaliphilus oremlandii (strain OhILAs)]	5	3	11	0	76.3	31.09
215	A8MIN1 Formate--tetrahydrofolate ligase [OS=Alkaliphilus oremlandii (strain OhILAs)]	5	2	4	0	59.8	9.83

216	P30721 Chaperone protein DnaK [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	4	2	4	0	65.6	6.43
217	A6TLJ1 60 kDa chaperonin [OS=Alkaliphilus metalliredigens (strain QYMF)]	7	2	3	0	57.9	7.71
218	B2TIR4 Ketol-acid reductoisomerase (NADP(+)) [OS=Clostridium botulinum (strain Eklund 17B / Type B)]	15	4	21	3	36.9	62.87
219	A6L048 L-fucose isomerase [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	8	4	8	1	65.6	20.55
220	A6LPQ4 DNA-directed RNA polymerase subunit beta [OS=Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)]	3	3	6	3	138.7	14.59
221	A6KYJ7 Elongation factor G [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 /	6	3	8	2	77.4	25.84

	NBRC 14291 / NCTC 11154)]						
222	A6LQ87 60 kDa chaperonin [OS=Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)]	9	3	4	1	57.5	9.98
223	C3KUC8 60 kDa chaperonin [OS=Clostridium botulinum (strain 657 / Type Ba4)]	9	3	5	0	57.9	12.44
224	Q97EH4 Elongation factor G [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	5	3	11	0	76.4	31.09
225	A6LPP6 Elongation factor Tu [OS=Clostridium beijerinckii (strain ATCC 51743 / NCIMB 8052)]	10	3	28	0	43.6	96.38
226	B1L1K0 60 kDa chaperonin [OS=Clostridium botulinum (strain Loch Maree / Type A3)]	9	3	5	0	57.9	12.44
227	A0PXT8 DNA-directed RNA polymerase subunit beta [OS=Clostridium novyi (strain NT)]	2	2	5	2	139.6	12.47
228	Q890N4 DNA-directed RNA polymerase subunit	2	2	5	2	138.8	12.47

	beta [OS=Clostridium tetani (strain Massachusetts / E88)]						
229	B9DYA1 DNA-directed RNA polymerase subunit beta [OS=Clostridium kluyveri (strain NBRC 12016)]	2	2	5	2	139	12.47
230	Q0TMN8 DNA-directed RNA polymerase subunit beta [OS=Clostridium perfringens (strain ATCC 13124 / DSM 756 / JCM 1290 / NCIMB 6125 / NCTC 8237 / Type A)]	2	2	5	2	138.5	12.47
231	A7GJ82 DNA-directed RNA polymerase subunit beta [OS=Clostridium botulinum (strain Langeland / NCTC 10281 / Type F)]	2	2	5	2	139.1	12.47
232	Q0SQD6 DNA-directed RNA polymerase subunit beta [OS=Clostridium perfringens (strain SM101 / Type A)]	2	2	5	2	138.5	12.47
233	B1IGG2 DNA-directed RNA polymerase subunit beta [OS=Clostridium	2	2	5	2	138.8	12.47

	botulinum (strain Okra / Type B1)]						
234	B1KSN3 DNA-directed RNA polymerase subunit beta [OS=Clostridium botulinum (strain Loch Maree / Type A3)]	2	2	5	2	139.8	12.47
235	A8MLD2 DNA-directed RNA polymerase subunit beta [OS=Alkaliphilus oremlandii (strain OhILAs)]	2	2	5	2	139	12.47
236	C1FMV9 DNA-directed RNA polymerase subunit beta [OS=Clostridium botulinum (strain Kyoto / Type A2)]	2	2	5	2	138.8	12.47
237	Q64NL1 50S ribosomal protein L2 [OS=Bacteroides fragilis (strain YCH46)]	10	2	6	2	29.7	17
238	Q8A5W2 Glucose-6-phosphate isomerase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	4	2	7	2	48.9	17.87
239	A5I7L4 DNA-directed RNA polymerase subunit beta [OS=Clostridium	2	2	5	2	138.8	12.47

	botulinum (strain Hall / ATCC 3502 / NCTC 13319 / Type A)]						
240	C3KVQ9 DNA-directed RNA polymerase subunit beta [OS=Clostridium botulinum (strain 657 / Type Ba4)]	2	2	5	2	140	12.47
241	A6L7P7 Glucose-6-phosphate isomerase [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	4	2	7	2	49	17.87
242	A6TWJ0 DNA-directed RNA polymerase subunit beta [OS=Alkaliphilus metalliredigens (strain QYMF)]	2	2	5	2	139.6	12.47
243	Q97EG9 DNA-directed RNA polymerase subunit beta [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	2	2	5	2	139.2	12.47
244	C4ZBU5 30S ribosomal protein S4 [OS=Agathobacter rectalis (strain ATCC 33656 / DSM 3377 /	9	2	5	2	22.7	11.72

	JCM 17463 / KCTC 5835 / VPI 0990)]						
245	A5N6M2 Chaperone protein DnaK [OS=Clostridium kluyveri (strain ATCC 8527 / DSM 555 / NCIMB 10680)]	3	2	3	2	66	7.3
246	O52631 Glyceraldehyde-3-phosphate dehydrogenase [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	4	2	6	2	35.8	17.04
247	A6L0A5 Polyribonucleotide nucleotidyltransferase [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	3	2	2	2	78.6	5.3
248	A6KYJ2 50S ribosomal protein L2 [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	10	2	6	2	29.6	17
249	A6TK65 ATP synthase subunit beta [OS=Alkaliphilus metalliredigens (strain QYMF)]	6	2	3	1	50.1	10.72

250	Q891G4 60 kDa chaperonin [OS=Clostridium tetani (strain Massachusetts / E88)]	6	2	3	1	58.4	7.79
251	Q0TN27 60 kDa chaperonin [OS=Clostridium perfringens (strain ATCC 13124 / DSM 756 / JCM 1290 / NCIMB 6125 / NCTC 8237 / Type A)]	6	2	3	1	57.3	8.87
252	Q877L9 Elongation factor Tu [OS=Clostridium tetani (strain Massachusetts / E88)]	8	2	24	1	43.4	85.68
253	P26821 60 kDa chaperonin [OS=Clostridium perfringens (strain 13 / Type A)]	6	2	3	1	57.3	8.87
254	Q890P3 30S ribosomal protein S3 [OS=Clostridium tetani (strain Massachusetts / E88)]	13	2	5	1	24.6	15.03
255	B1IE34 ATP synthase subunit beta [OS=Clostridium botulinum (strain Okra / Type B1)]	5	2	6	0	50.7	18.1
256	B1KSS8 ATP synthase subunit beta [OS=Clostridium botulinum (strain Loch Maree / Type A3)]	5	2	6	0	50.7	18.1
257	B2TIG9 DNA-directed RNA	2	2	6	0	131.3	16.29

	polymerase subunit beta' [OS=Clostridium botulinum (strain Eklund 17B / Type B)]						
258	A6KYK2 DNA-directed RNA polymerase subunit beta' [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	1	2	6	0	158.7	16.29
259	Q8A9V4 ATP synthase subunit beta [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	5	2	6	0	55.3	18.1
260	B1IGF7 Elongation factor G [OS=Clostridium botulinum (strain Okra / Type B1)]	3	2	7	0	76.4	19.93
261	B2UYA4 DNA-directed RNA polymerase subunit beta' [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	2	2	6	0	131	16.29
262	A6LPQ5 DNA-directed RNA polymerase subunit beta' [OS=Clostridium	2	2	6	0	131.7	16.29

	beijerinckii (strain ATCC 51743 / NCIMB 8052)]						
263	A0PXT9 DNA-directed RNA polymerase subunit beta' [OS=Clostridium novyi (strain NT)]	2	2	6	0	132.4	16.29
264	C3KVQ4 Elongation factor G [OS=Clostridium botulinum (strain 657 / Type Ba4)]	3	2	7	0	76.4	19.93
265	C1FQP5 ATP synthase subunit beta [OS=Clostridium botulinum (strain Kyoto / Type A2)]	5	2	6	0	50.8	18.1
266	A8MLD3 DNA-directed RNA polymerase subunit beta' [OS=Alkaliphilus oremlandii (strain OhILAs)]	2	2	6	0	132	16.29
267	B1KSN2 DNA-directed RNA polymerase subunit beta' [OS=Clostridium botulinum (strain Loch Maree / Type A3)]	2	2	6	0	131.5	16.29
268	A7FQH9 ATP synthase subunit beta [OS=Clostridium botulinum (strain ATCC 19397 / Type A)]	5	2	6	0	50.8	18.1
269	A5N4P0 DNA-directed RNA	2	2	6	0	131.4	16.29

	polymerase subunit beta' [OS=Clostridium kluyveri (strain ATCC 8527 / DSM 555 / NCIMB 10680)]						
270	A5I7L3 DNA-directed RNA polymerase subunit beta' [OS=Clostridium botulinum (strain Hall / ATCC 3502 / NCTC 13319 / Type A)]	2	2	6	0	131.5	16.29
271	Q9Z687 ATP synthase subunit beta [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	5	2	6	0	51.1	18.1
272	A8MJV9 ATP synthase subunit beta [OS=Alkaliphilus oremlandii (strain OhILAs)]	5	2	6	0	49.9	18.1
273	B9DYA6 Elongation factor G [OS=Clostridium kluyveri (strain NBRC 12016)]	3	2	7	0	76.4	19.8
274	P0C2E8 DNA-directed RNA polymerase subunit beta' [OS=Clostridium perfringens (strain 13 / Type A)]	2	2	6	0	131.5	16.29

275	A7FZ72 Elongation factor G [OS=Clostridium botulinum (strain ATCC 19397 / Type A)]	3	2	7	0	76.3	19.93
276	B1IGG1 DNA-directed RNA polymerase subunit beta' [OS=Clostridium botulinum (strain Okra / Type B1)]	2	2	6	0	131.6	16.29
277	A6L4L7 ATP synthase subunit beta [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	5	2	6	0	56.1	18.1
278	B1KSM8 Elongation factor G [OS=Clostridium botulinum (strain Loch Maree / Type A3)]	3	2	7	0	76.3	19.93
279	B2TK00 ATP synthase subunit beta [OS=Clostridium botulinum (strain Eklund 17B / Type B)]	5	2	6	0	50.3	18.1
280	B2UZK0 ATP synthase subunit beta [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	5	2	6	0	50.3	18.1
281	Q8A470 DNA-directed RNA polymerase subunit	1	2	6	0	158.3	16.29

	beta' [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]						
282	A5N3H7 ATP synthase subunit beta [OS=Clostridium kluyveri (strain ATCC 8527 / DSM 555 / NCIMB 10680)]	5	2	6	0	50.7	18.1
283	A0Q2Z4 ATP synthase subunit beta [OS=Clostridium novyi (strain NT)]	5	2	6	0	51	18.1
284	A7GJ81 DNA-directed RNA polymerase subunit beta' [OS=Clostridium botulinum (strain Langeland / NCTC 10281 / Type F)]	2	2	6	0	131.6	16.29
	FASP						
1	P02769 Albumin [OS=Bos taurus]	12	8	15	8	69.2	39.26
2	Q2UVX4 Complement C3 [OS=Bos taurus]	3	5	13	5	187.1	29.92
3	Q7SIH1 Alpha-2-macroglobulin [OS=Bos taurus]	4	5	15	5	167.5	40.48
4	Q3T166 Mucosal pentraxin [OS=Bos taurus]	17	4	24	4	24.7	68.4
5	P15396 Ectonucleotide pyrophosphatase/phosphodiesterase family	5	4	15	4	99.5	42.68

	member 3 [OS=Bos taurus]						
6	P13214 Annexin A4 [OS=Bos taurus]	10	3	9	3	35.9	23.35
7	P60712 Actin, cytoplasmic 1 [OS=Bos taurus]	9	3	6	3	41.7	14.52
8	P34955 Alpha-1-antiproteinase [OS=Bos taurus]	5	2	8	2	46.1	21.29
9	P06394 Keratin, type I cytoskeletal 10 [OS=Bos taurus]	4	2	2	2	54.8	4.82
10	P05805 Proproteinase E [OS=Bos taurus]	8	2	8	2	27.3	20.51
11	P79098 Aminopeptidase N [OS=Bos taurus]	2	2	8	2	109.2	21.8
12	P00760 Cationic trypsin [OS=Bos taurus]	13	2	7	2	25.8	19.41
13	P13753 BOLA class I histocompatibility antigen, alpha chain BL3-7 [OS=Bos taurus]	7	2	3	2	41.5	8.38
14	Q8SPP7 Peptidoglycan recognition protein 1 [OS=Bos taurus]	13	2	8	2	21.1	20.24
15	P63258 Actin, cytoplasmic 2 [OS=Bos taurus]	9	3	6	3	41.8	14.52
16	P06293 Serpin-Z4 [OS=Hordeum vulgare]	11	4	13	4	43.2	33.99
17	Q43492 Serpin-Z7 [OS=Hordeum vulgare]	8	3	7	3	42.8	16.35
18	P32936 Alpha-amylase/trypsin inhibitor CMb	18	2	4	2	16.5	9.61

	[OS=Hordeum vulgare]						
19	B2UYT8 Ketol-acid reductoisomerase (NADP(+)) [OS=Clostridium botulinum (strain Alaska E43 / Type E3)]	13	4	8	3	37	19.94
20	P94316 NAD-specific glutamate dehydrogenase [OS=Bacteroides fragilis (strain YCH46)]	7	3	9	3	48.4	22.16
21	A6L050 2,3-bisphosphoglycerate-independent phosphoglycerate mutase [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	4	3	6	3	55.8	16.67
22	Q59309 Glyceraldehyde-3-phosphate dehydrogenase [OS=Clostridium pasteurianum]	6	3	9	3	36.1	23.84
23	C4ZBL1 Phosphoenolpyruvate carboxykinase (ATP) [OS=Agathobacter rectalis (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)]	7	3	12	2	59	31.16
24	Q8A463 Elongation factor Tu	9	3	8	2	43.6	23.54

	[OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]						
25	C4Z0Q6 Phosphoenolpyruvate carboxykinase (ATP) [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]	8	3	10	2	59	27.57
26	P94598 Glutamate dehydrogenase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	7	3	6	2	49	13.76
27	Q9EZ02 Pyrophosphate--fructose 6-phosphate 1-phosphotransferase [OS=Spirochaeta thermophila (strain ATCC 49972 / DSM 6192 / RI 19.B1)]	4	2	10	2	61	29.79
28	Q8A490 30S ribosomal protein S8 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	16	2	5	2	14.6	13.93
29	A9KRZ3 Elongation factor G [OS=Lachnoclostridium phytofermentans	4	2	2	2	78.1	4.59

	(strain ATCC 700394 / DSM 18823 / ISDg)]						
30	Q46149 Toxin A [OS=Clostridium novyi]	1	2	6	2	250	14.87
31	A0PXT9 DNA-directed RNA polymerase subunit beta' [OS=Clostridium novyi (strain NT)]	2	2	5	2	132.4	8.54
32	Q8A5W4 Lysine--tRNA ligase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	4	2	3	2	65.9	6.69
33	Q8A474 Elongation factor G [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	3	2	5	2	77.5	11.41
34	Q8A4N6 Polyribonucleotide nucleotidyltransferase [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	3	2	6	2	78.3	16.25
35	Q8A499 30S ribosomal protein S13 [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 /	14	2	5	2	14.1	14.49

	DSM 2079 / NCTC 10582 / E50 / VPI-5482)]						
36	Q64PM7 Glucose-6-phosphate isomerase [OS=Bacteroides fragilis (strain YCH46)]	4	2	5	2	48.7	10.87
37	A9KRZ4 Elongation factor Tu [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	7	2	4	1	43.8	13.29
38	A9KQ65 Ketol-acid reductoisomerase (NADP(+)) [OS=Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg)]	8	2	8	1	36.7	25.25
39	Q8A414 Phosphoenolpyruvate carboxykinase (ATP) [OS=Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)]	5	2	13	1	59.1	34.5
40	P95544 NAD(P)-specific glutamate dehydrogenase [OS=Prevotella ruminicola]	5	2	7	1	48.8	17.28
41	Q0TMN0 Elongation factor Tu [OS=Clostridium perfringens (strain ATCC 13124 / DSM	6	2	4	1	43.5	12.88

	756 / JCM 1290 / NCIMB 6125 / NCTC 8237 / Type A)]						
42	P33165 Elongation factor Tu [OS=Bacteroides fragilis (strain YCH46)]	9	3	8	2	43.6	23.54
43	A6KYK9 Elongation factor Tu [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	9	3	8	2	43.5	23.54
44	Q5L8C3 30S ribosomal protein S8 [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	16	2	5	2	14.6	13.93
45	Q5L7Z7 Polyribonucleotide nucleotidyltransferase [OS=Bacteroides fragilis (strain ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)]	3	2	6	2	78.3	16.25
46	A6KYI1 30S ribosomal protein S8 [OS=Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / JCM 5826 / NBRC 14291 / NCTC 11154)]	16	2	5	2	14.6	13.93
47	C4ZB99 Elongation factor Tu	6	2	4	1	43.4	12.88

	[OS=Agathobacter rectalis (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)]						
48	C4Z2R9 Elongation factor Tu [OS=Lachnospira eligens (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)]	7	2	4	0	44	13.29
49	O52631 Glyceraldehyde-3-phosphate dehydrogenase [OS=Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)]	4	2	6	2	35.8	17.61
50	B2TIR4 Ketol-acid reductoisomerase (NADP(+)) [OS=Clostridium botulinum (strain Eklund 17B / Type B)]	6	2	5	1	36.9	12.16

Appendix Table 5-1. Biological processes of faecal microbial proteins with at least two peptides involved.

No	Peptides	GO Term	Biological process
Bacteria			
1	40	GO:0006094	Gluconeogenesis
2	38	GO:0006096	Glycolytic process
3	36	GO:0006520	Cellular amino acid metabolic process
4	34	GO:0006006	Glucose metabolic process
5	30	GO:0005975	Carbohydrate metabolic process
6	29	GO:0006412	Translation
7	20	GO:0006355	Regulation of transcription, DNA-templated
8	17	GO:0006090	Pyruvate metabolic process
9	14	GO:0006281	DNA repair
10	12	GO:0030388	Fructose 1,6-bisphosphate metabolic process
11	11	GO:0006260	DNA replication
12	11	GO:0045454	Cell redox homeostasis
13	10	GO:0009252	Peptidoglycan biosynthetic process
14	9	GO:0016226	Iron-sulfur cluster assembly
15	9	GO:0009306	Protein secretion
16	8	GO:0035999	Tetrahydrofolate interconversion
17	7	GO:0055085	Transmembrane transport
18	7	GO:0006306	DNA methylation
19	7	GO:0006351	Transcription, DNA-templated
20	7	GO:0055072	Iron ion homeostasis
21	7	GO:0000160	Phosphorelay signal transduction system
22	7	GO:0009097	Isoleucine biosynthetic process
23	6	GO:0032259	Methylation
24	6	GO:0006265	DNA topological change
25	6	GO:0009432	SOS response
26	6	GO:0009099	Valine biosynthetic process
27	6	GO:0071973	Bacterial-type flagellum-dependent cell motility
28	6	GO:0006002	Fructose 6-phosphate metabolic process
29	5	GO:0035556	Intracellular signal transduction
30	5	GO:0071555	Cell wall organization
31	5	GO:0009307	DNA restriction-modification system
32	5	GO:0015031	Protein transport
33	5	GO:0015074	DNA integration
34	5	GO:0006310	DNA recombination
35	5	GO:0006457	Protein folding
36	4	GO:0006084	Acetyl-CoA metabolic process
37	4	GO:0051301	Cell division

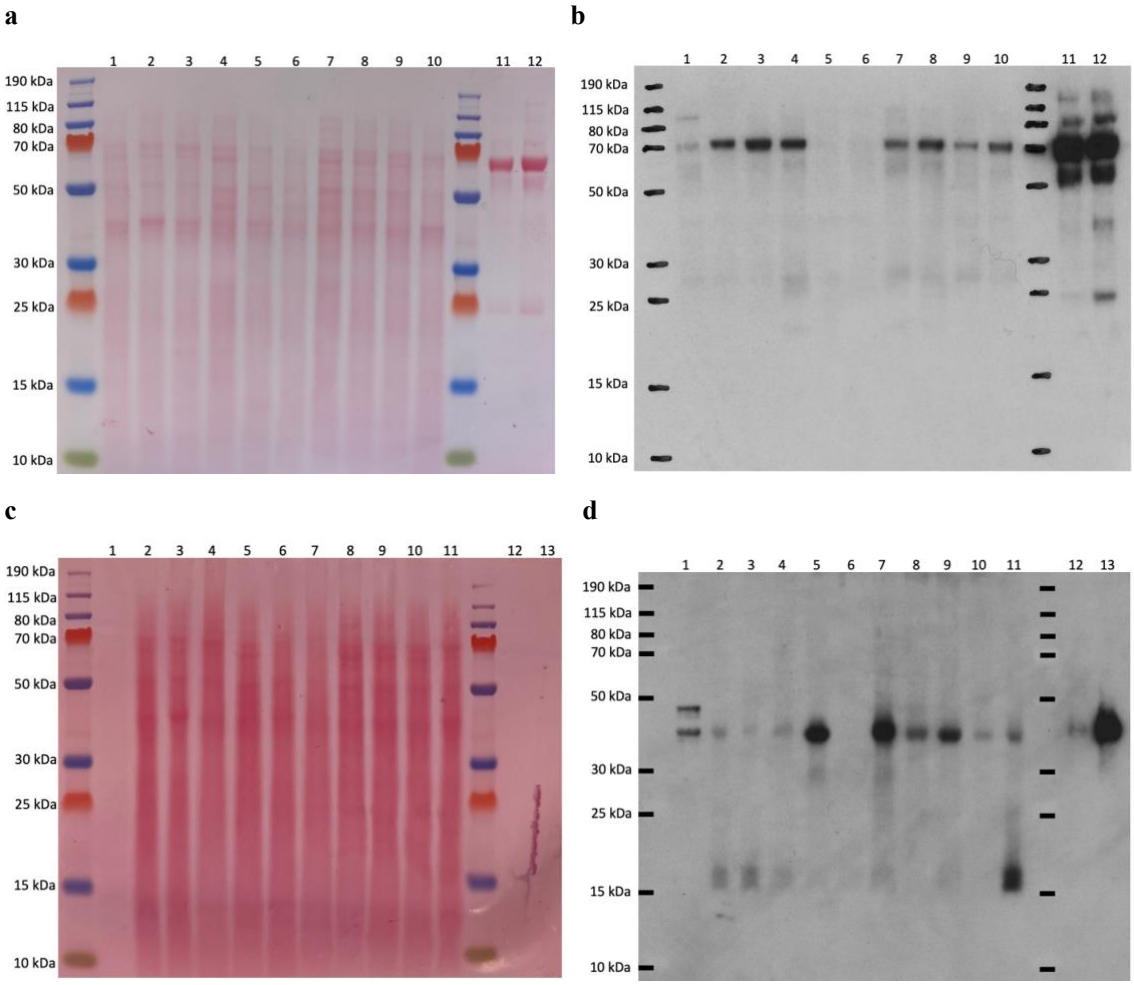
38	4	GO:0006261	DNA-dependent DNA replication
39	4	GO:0006289	Nucleotide-excision repair
40	4	GO:0022900	Electron transport chain
41	3	GO:0006352	DNA-templated transcription, initiation
42	3	GO:0006396	RNA processing
43	3	GO:0006886	Intracellular protein transport
44	3	GO:0006541	Glutamine metabolic process
45	3	GO:0006189	'de novo' IMP biosynthetic process
46	3	GO:0007155	Cell adhesion
47	3	GO:0006313	Transposition, DNA-mediated
48	3	GO:0007165	Signal transduction
49	3	GO:0030163	Protein catabolic process
50	3	GO:0006897	Endocytosis
51	3	GO:0009058	Biosynthetic process
52	3	GO:0006633	Fatty acid biosynthetic process
53	3	GO:0009073	Aromatic amino acid family biosynthetic process
54	3	GO:0008652	Cellular amino acid biosynthetic process
55	3	GO:0009423	Chorismate biosynthetic process
56	3	GO:0042355	L-fucose catabolic process
57	3	GO:0009253	Peptidoglycan catabolic process
58	3	GO:0006108	Malate metabolic process
59	3	GO:0006629	Lipid metabolic process
60	3	GO:0019605	Butyrate metabolic process
61	3	GO:0006188	IMP biosynthetic process
62	3	GO:0048741	Skeletal muscle fiber development
63	3	GO:0048488	Synaptic vesicle endocytosis
64	3	GO:0090131	Mesenchyme migration
65	3	GO:0010628	Positive regulation of gene expression
66	3	GO:0014829	Vascular associated smooth muscle contraction
67	3	GO:0060047	Heart contraction
68	3	GO:0030240	Skeletal muscle thin filament assembly
69	2	GO:0009113	Purine nucleobase biosynthetic process
70	2	GO:0006817	Phosphate ion transport
71	2	GO:0030036	Actin cytoskeleton organization
72	2	GO:0051028	MRNA transport
73	2	GO:0045226	Extracellular polysaccharide biosynthetic process
74	2	GO:0006402	MRNA catabolic process
75	2	GO:0000281	Mitotic cytokinesis
76	2	GO:0008616	Queuosine biosynthetic process
77	2	GO:0046677	Response to antibiotic
78	2	GO:2001295	Malonyl-CoA biosynthetic process
79	2	GO:0008152	Metabolic process

80	2	GO:0006207	'de novo' pyrimidine nucleobase biosynthetic process
81	2	GO:0044205	'de novo' UMP biosynthetic process
82	2	GO:0006424	Glutamyl-tRNA aminoacylation
83	2	GO:0006811	Ion transport
84	2	GO:0006268	DNA unwinding involved in DNA replication
85	2	GO:0007018	Microtubule-based movement
86	2	GO:0015833	Peptide transport
87	2	GO:0000105	Histidine biosynthetic process
88	2	GO:0005980	Glycogen catabolic process
89	2	GO:0071704	Organic substance metabolic process
90	2	GO:0006430	Lysyl-tRNA aminoacylation
91	2	GO:0008299	Isoprenoid biosynthetic process
92	2	GO:0006730	One-carbon metabolic process
93	2	GO:0006099	Tricarboxylic acid cycle
94	2	GO:0006436	Tryptophanyl-tRNA aminoacylation
95	2	GO:0019877	Diaminopimelate biosynthetic process
96	2	GO:0009089	Lysine biosynthetic process via diaminopimelate
97	2	GO:0016042	Lipid catabolic process
98	2	GO:0006013	Mannose metabolic process
99	2	GO:0009098	Leucine biosynthetic process
100	2	GO:0008360	Regulation of cell shape
101	2	GO:0046373	L-arabinose metabolic process
102	2	GO:0016567	Protein ubiquitination
103	2	GO:0006511	Ubiquitin-dependent protein catabolic process
104	2	GO:0042026	Protein refolding
105	2	GO:0043419	Urea catabolic process
106	2	GO:0030476	Ascospore wall assembly
107	2	GO:0034599	Cellular response to oxidative stress
108	2	GO:0030010	Establishment of cell polarity
109	2	GO:1902404	Mitotic actomyosin contractile ring contraction
110	2	GO:0000011	Vacuole inheritance
111	2	GO:0006631	Fatty acid metabolic process
112	2	GO:0033275	Actin-myosin filament sliding
Archaea			
1	26	GO:0005975	Carbohydrate metabolic process
2	26	GO:0006412	Translation
3	24	GO:0006355	Regulation of transcription, DNA-templated
4	19	GO:0006281	DNA repair
5	18	GO:0006260	DNA replication
6	12	GO:0006265	DNA topological change
7	12	GO:0032259	Methylation

8	12	GO:0006310	DNA recombination
9	11	GO:0055085	Transmembrane transport
10	9	GO:0006520	Cellular amino acid metabolic process
11	9	GO:0006457	Protein folding
12	9	GO:0007165	Signal transduction
13	9	GO:0000160	Phosphorelay signal transduction system
14	9	GO:0015074	DNA integration
15	9	GO:0006541	Glutamine metabolic process
16	8	GO:0051301	Cell division
17	8	GO:0009432	SOS response
18	7	GO:0015948	Methanogenesis
19	7	GO:0015031	Protein transport
20	7	GO:0006189	'de novo' IMP biosynthetic process
21	7	GO:0009058	Biosynthetic process
22	6	GO:0006261	DNA-dependent DNA replication
23	6	GO:0006302	Double-strand break repair
24	6	GO:0007049	Cell cycle
25	6	GO:0009405	Obsolete pathogenesis
26	6	GO:0006289	Nucleotide-excision repair
27	5	GO:0006813	Potassium ion transport
28	5	GO:0006090	Pyruvate metabolic process
29	5	GO:0006268	DNA unwinding involved in DNA replication
30	5	GO:0007155	Cell adhesion
31	5	GO:0009252	Peptidoglycan biosynthetic process
32	5	GO:0044780	Bacterial-type flagellum assembly
33	5	GO:0006782	Protoporphyrinogen IX biosynthetic process
34	4	GO:0009097	Isoleucine biosynthetic process
35	4	GO:0009099	Valine biosynthetic process
36	4	GO:0006306	DNA methylation
37	4	GO:0097056	Selenocysteinyl-tRNA(Sec) biosynthetic process
38	4	GO:0006304	DNA modification
39	4	GO:0000272	Polysaccharide catabolic process
40	4	GO:0051607	Defense response to virus
41	4	GO:0006313	Transposition, DNA-mediated
42	4	GO:0006428	Isoleucyl-tRNA aminoacylation
43	4	GO:0006096	Glycolytic process
44	4	GO:0006099	Tricarboxylic acid cycle
45	4	GO:0071555	Cell wall organization
46	4	GO:0006526	Arginine biosynthetic process
47	3	GO:0016226	Iron-sulfur cluster assembly
48	3	GO:0035999	Tetrahydrofolate interconversion
49	3	GO:0009082	Branched-chain amino acid biosynthetic process

50	3	GO:0009098	Leucine biosynthetic process
51	3	GO:0016260	Selenocysteine biosynthetic process
52	3	GO:0006434	Seryl-tRNA aminoacylation
53	3	GO:0016539	Intein-mediated protein splicing
54	3	GO:0071897	DNA biosynthetic process
55	3	GO:0006270	DNA replication initiation
56	3	GO:0006935	Chemotaxis
57	3	GO:0006094	Gluconeogenesis
58	3	GO:0032196	Transposition
59	3	GO:0006006	Glucose metabolic process
60	3	GO:0008360	Regulation of cell shape
61	3	GO:0071973	Bacterial-type flagellum-dependent cell motility
62	3	GO:0006825	Copper ion transport
63	3	GO:0008033	TRNA processing
64	3	GO:0006614	SRP-dependent cotranslational protein targeting to membrane
65	3	GO:0017004	Cytochrome complex assembly
66	3	GO:0009306	Protein secretion
67	3	GO:0009307	DNA restriction-modification system
68	3	GO:0000724	Double-strand break repair via homologous recombination
69	2	GO:0016567	Protein ubiquitination
70	2	GO:0016310	Phosphorylation
71	2	GO:0006269	DNA replication, synthesis of RNA primer
72	2	GO:0042026	Protein refolding
73	2	GO:0006396	RNA processing
74	2	GO:0016192	Vesicle-mediated transport
75	2	GO:0045087	Innate immune response
76	2	GO:0006814	Sodium ion transport
77	2	GO:0044210	'de novo' CTP biosynthetic process
78	2	GO:0002098	TRNA wobble uridine modification
79	2	GO:0006400	TRNA modification
80	2	GO:0006325	Chromatin organization
81	2	GO:0009234	Menaquinone biosynthetic process
82	2	GO:0008616	Queuosine biosynthetic process
83	2	GO:0006351	Transcription, DNA-templated
84	2	GO:0009236	Cobalamin biosynthetic process
85	2	GO:0008652	Cellular amino acid biosynthetic process
86	2	GO:0042773	ATP synthesis coupled electron transport
87	2	GO:0006429	Leucyl-tRNA aminoacylation
88	2	GO:0006511	Ubiquitin-dependent protein catabolic process
89	2	GO:0006486	Protein glycosylation
90	2	GO:0045104	Intermediate filament cytoskeleton organization
91	2	GO:0006897	Endocytosis

92	2	GO:0019430	Removal of superoxide radicals
93	2	GO:0035556	Intracellular signal transduction
94	2	GO:0019354	Siroheme biosynthetic process
95	2	GO:0006275	Regulation of DNA replication
96	2	GO:0046677	Response to antibiotic
97	2	GO:0006633	Fatty acid biosynthetic process
98	2	GO:0006352	DNA-templated transcription, initiation
99	2	GO:0070966	Nuclear-transcribed mRNA catabolic process, no-go decay
100	2	GO:0070481	Nuclear-transcribed mRNA catabolic process, non-stop decay
101	2	GO:0071025	RNA surveillance
102	2	GO:0009399	Nitrogen fixation
103	2	GO:0043335	Protein unfolding
104	2	GO:0019427	Acetyl-CoA biosynthetic process from acetate
105	2	GO:0007156	Homophilic cell adhesion via plasma membrane adhesion molecules
106	2	GO:0030001	Metal ion transport



Appendix Figure 5-1. Full image of blot of (a) and (b) albumin and (c) and (d) serpin Z4; (a) and (c) are stained by ponceau S, (b) and (d) are visualized using ECL reaction.

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