Journal of Proteomics 285 (2023) 104941

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Quantitative TMT-based proteomics revealing host, dietary and microbial proteins in bovine faeces including barley serpin Z4, a prominent component in the head of beer

Y. Huang [a,1], N.N. Jonsson [a], M. McLaughlin [a], R. Burchmore [b], P.C.D. Johnson [a], R.O. Jones [a], S. McGill [b], N. Brady [a], S. Weidt [b], P.D. Eckersall [a,c,*]

[a] School of Biodiversity, One Health & Veterinary Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, G61 1QH, UK
[b] Institute of Infection, Immunity & Inflammation and Glasgow Polyomics, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G61 1BD, UK
[c] Interdisciplinary Laboratory of Clinical Analysis of the University of Murcia (Interlab-UMU), Department of Animal Medicine and Surgery, Veterinary School, University of Murcia, Murcia 30100, Spain

ARTICLE INFO

Keywords:
Beef cattle
Faecal proteome
TMT
Bovine glycoprotein
Barley serpin
Microbiota

ABSTRACT

There has been little information about the proteome of bovine faeces or about the contribution to the faecal proteome of proteins from the host, the feed or the intestinal microbiome. Here, the bovine faecal proteome and the origin of its component proteins was assessed, while also determining the effect of treating barley, the major carbohydrate in the feed, with either ammonia (ATB) or sodium propionate (PTB) preservative. Healthy continental crossbreed steers were allocated to two groups and fed on either of the barley-based diets. Five faecal samples from each group were collected on Day 81 of the trial and analysed by quantitative proteomics using nLC-ESI-MS/MS after tandem mass tag labelling. In total, 281 bovine proteins, 199 barley proteins, 176 bacterial proteins and 190 archaeal proteins were identified in the faeces. Mucosal pentraxin, albumin and digestive enzymes were among bovine proteins identified. Serpin Z4 a protease inhibitor was the most abundant barley protein identified which is also found in barley-based beer, while numerous microbial proteins were identified, many originating bacteria from *Clostridium*, while *Methanobrevibacter* was the dominant archaeal genus. Thirty-nine proteins were differentially abundant between groups, the majority being more abundant in the PTB group compared to the ATB group.

Significance: Proteomic examination of faeces is becoming a valuable means to assess the health of the gastrointestinal tract in several species, but knowledge on the proteins present in bovine faeces is limited. This investigation aimed to characterise the proteome of bovine faecal extracts in order to evaluate the potential for investigations of the proteome as a means to assess the health, disease and welfare of cattle in the future. The investigation was able to identify proteins in bovine faeces that had been (i) produced by the individual cattle, (ii) present in the barley-based feed eaten by the cattle or (iii) produced by bacteria and other microbes in the rumen or intestines. Bovine proteins identified included mucosal pentraxin, serum albumin and a variety of digestive enzymes. Barley proteins found in the faeces included serpin Z4, a protease inhibitor that is also found in beer having survived the brewing process. Bacterial and archaeal proteins in the faecal extracts were related to several pathways related to the metabolism of carbohydrates. The recognition of the range of proteins that can be identified in bovine faeces raises the possibility that non-invasive sample collection of this material could provide a novel diagnostic approach to cattle health and welfare.

* Corresponding author at: School of Biodiversity One Health & Veterinary Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, G61 1QH, UK.
E-mail address: David.Eckersall@glasgow.ac.uk (P.D. Eckersall).
[1] Present address: Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China.

https://doi.org/10.1016/j.jprot.2023.104941
Received 1 March 2023; Received in revised form 21 May 2023; Accepted 25 May 2023
Available online 5 June 2023

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. The colour, odour, shape, consistency of faeces provides information about the state of the host [1,2] dietary information, behaviour and physiology [3], and the interactions between the host and the microbiota [4]. 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 [5], because some 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 because they are relatively less diluted by circulation in blood or lymphatics. Proteomic analysis of faeces may complement metagenomic analyses, particularly for components derived from diet, where DNA is likely degraded after transit through the digestive system. Host protein markers in faeces may correlate with disease.

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 [6], colorectal cancer [7] and cystic fibrosis [8]. Faecal proteomes in mouse [9], monkey [3], dog [2] and sheep [10] have also been studied. However, much of the research on bovine faeces has focused on the microbiota [4], 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. Recently multiplex isobaric labelling approaches, including tandem mass tagging (TMT) have been applied to investigations in animal and veterinary science [2,11,12], enabling qualitative and quantitative analysis of multiple sample protein groups at the same time reducing sample handling and inter-analysis variability. The advantages of a TMT proteomics approach were applied in the present study.

From our previous studies, 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 this treatment [1]. Furthermore it was found that the treatment of barley with sodium propionate to preserve the feed at low pH, increased the faecal starch concentration and proportion of animals with diarrhoea in animals compared to those fed the ammonia-treated barley-based diet [13]. In following up on the differences in faecal starch and diarrhoea incidence, this proteomic investigation was designed to assess the effects of these diets on the faecal proteome, including an initial step to refine the methods of sample processing to improve rates of protein identification as described in Huang et al. [14]. For this investigation, TMT-based proteomics was applied to (1) characterise the bovine, barley and microbial proteomes in the bovine faeces and to (2) 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 to inform the development of future studies to optimize diets for cattle.

2. Methods

2.1. Ethics statement

This study 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) as the study only required non-invasive bovine faecal sample collection. All the data 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.

2.2. Animals and treatments

The faecal samples ($n = 10$) used in the investigation were collected from a larger study of two hundred and seventeen continental crossbreed steers (predominantly Limousin and Charolais; 506 ± 82 days old, 481 ± 38 kg) housed on 27/07/2017 [13]. All animals were treated on arrival on farm against parasites using anthelmintic products (ivermectin and nitroxynil) and were vaccinated against infectious bovine rhinotracheitis. The animals were allocated to 4 pens (2 pens/treatment group) after stratification on age and weight, and were fed three transition diets for 12 or 18 days before receiving one of two different diets for 114 ± 10 days (Supplementary Table S1), ammonia-treated barley-based diet (ATB, 93 cattle) or sodium propionate-treated barley-based diet (PTB, 124 cattle). The barley in both ATB and PTB were prepared by Harbro Limited according to their standard recommended protocol (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, 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. The inclusion rate of sodium propionate (Prograin, Harbro Limited, Turriff, Scotland) varied from 6.5 to 7.5 L/t of grain, depending on the moisture content of the grain. Total-mixed rations and all dietary components were analysed by NIR (Foss-NIRSystems 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.

2.3. Sample collection and protein extraction

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 >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, the 5 highest volume faecal samples that were scored either 1 or 2 (i.e., from cattle that had well-digested fibre and no diarrhoea) were selected from each group from the collection on Day-81. The selected faecal samples came from different animals.

The protein extraction method was modified from our previous study [14]. 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, Irvine, USA). One mini protease inhibitor cocktail tablet (Sigma, Welwyn Garden City, 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 1000 ×*g* for 5 min at 4 °C, the supernatants from the replicates of each sample were collected and pooled, while the residual solid material was subjected to bead beating for a second time and their supernatants combined with that from the first bead-beating. The supernatant from the faecal extract of each sample was sonicated (VC-130 Ultrasonic liquid

processor, Sonics & Materials, Newtown, USA) 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, Poole, UK), centrifuging at 3200 × *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 sample 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, Welwyn Garden City, UK) for one hour and de-stained in 7.5% acetic acid with 20% methanol overnight.

2.4. TMT labelling and LC-MS/MS

The faecal samples were processed further by an in-gel sample preparation (IGSP) method as described in Huang et al. [14]. Briefly, the extracted protein samples were run on 10% polyacrylamide gels for only a short time (5 min) before staining with Coomassie blue, such that all proteins were in a narrow gel section and enabled excision of all proteins from 10 to 190 kDa in a single gel piece, removing large molecules such as high molecular weight (MW) glycoproteins or oligosaccharides as part of the clean-up process of the faecal samples. Proteins were digested in-gel by porcine trypsin (Thermo Scientific, UK) to obtain peptides for labelling with TMT conjugates. 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 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 [14], 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 min 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 resolving power (RP) (over a mass range of 380–1800 *m/z*) followed by isolation and collision-induced dissociation (CID) fragmentation the top 3 precursor ions from the MS scan in the linear ion trap. The three precursor ions were also subjected to higher-energy collisional dissociation (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.

2.5. Protein identification and quantification

2.5.1. Protein identification and relative quantification between ATB and PTB groups

Protein identification and relative quantification between ATB and PTB groups 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 and in relatively large quantities [4], 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.

2.5.2. Protein identification and relative quantification between proteins

Protein quantification and relative quantification between proteins was performed by determining the exponential modified protein abundance index (emPAI) with MS data resulting from the analysis of the pooled TMT sample processed using the automated Matrix Science Mascot Daemon server (v2.6.2). The emPAI was used to provide an estimate of relative protein abundance between individual proteins in the pooled sample made by mixing all TMT labelled samples ($n = 10$) including faecal extracts from all ATB and PTB cows. Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the Swissprot databases restricting the search to i) bovine (*Bos taurus*), ii) barley (*Hordeum vulgare*) taxonomies and in the NCBI databases while restricting the search for microbial proteins to iii) *Clostridium* and iv) *Methanobrevibacter* taxonomies and allowing a mass tolerance of 0.6 Da for both MS and MS/MS analyses. In addition, carbamidomethyl (C) was set as a fixed modification and oxidation (M), Iodo (Y), TMT6plex (K) and TMT6plex (N-term) were allowed as variable modifications. The emPAI for each protein was recorded for comparison of abundance between individual proteins in the pooled sample used for TMT analysis.

2.6. Validation of proteomics by Western blot

To validate the results of the proteomes, the faecal samples were subjected to Western blot (WB) analysis of two candidate proteins making use of antibodies that were available for use: a rabbit polyclonal antibody to bovine serum albumin-HRP (antibodies-online GmbH, Germany), and an antibody to barley serpin Z4 (a gift from Professor Greg Tanner, University of Melbourne, Australia) [15]. 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 WB of albumin; for serpin Z4, 400 ng of barley protein (extracted from untreated barley on farm) was used as control, and for further comparison 200 ng and 400 ng of protein from a Scottish barley-based beer (Original Best, Belhaven Brewery, Belhaven, UK) were also tested, as barley serpins are present in beer [16]. 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 concentration on an Amicon Ultra-0.5 centrifugal filter unit with 10 kDa cut-off (Merck, Poole, UK). After electrophoresis, the gels were rinsed in water and blotted to nitrocellulose transfer membranes (Invitrogen, UK) using an iBlot™ 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. Antibodies to serum albumin (already conjugated to HRP) and to serpin Z4 were

added at dilution of 1:1000 and 1:2000 respectively, 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, Buckinghamshire, UK) after this wash, while the membrane for serpin Z4 was incubated with 1: 3000 of goat anti-rabbit IgG HRP (Abcam, Cambridge, UK) for 1 h before three-time TBST washes, ECL reaction and visualization.

2.7. Data analysis for differentially abundant proteins (DAP)

Statistical analyses and graphical presentation of the results were mainly performed in R version 4.0.3 [17]. Relative quantitation of protein abundance between ATP and PTB groups was achieved as a result of TMT labelling, 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 $p < 0.05$ and fold change (FC) of ATB to PTB > 1.5 or FC of PTB to ATB > 1.5. 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 [18] (https://unipept.ugent.be). The analysis of protein band intensity of WB was quantified using ImageJ software, and the differences in proteins between groups were tested using Mann-Whitney-Wilcoxon tests.

3. Results

3.1. Proteomics

Clear faecal protein bands could be seen from the Coomassie blue-stained SDS-PAG gel (Fig. 1), which showed successful extraction of proteins from all samples. The protein bands in the range of 30 kDa - 190 kDa were expected to be high abundance proteins, but there were no clearly differentiated protein band patterns between ATB and PTB groups. In total, 281 bovine proteins, 199 barley proteins, 176 bacterial proteins and 190 archaeal proteins were identified in the bovine faeces

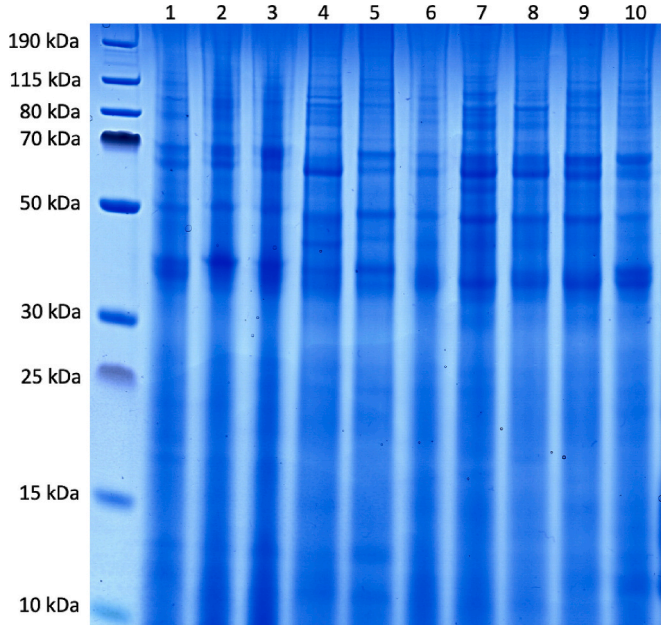

Fig. 1. Bovine faecal proteins shown on a 10% Bis-Tris gel by Coomassie blue staining. Lanes 1–5 are samples from ATB group and lanes 6–10 are samples from PTB group.

by TMT-based nUHPLC-ESI-MS/MS and were used for further analyses. The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE [19] partner repository with the data set identifier PXD036027.

The 20 proteins from each of the host, barley, bacteria and archaea databases in the faeces that had the highest relative abundances within each diet group are presented in Fig. 2 and the proteins that were significant DAP between the ATB and PTB diet groups are shown in Table 1. The 10 faecal samples were plotted using their scores in principal component 1 (PC1: 25.62%) and principal component 2 (PC2: 20.11%) (Fig. 3a). No obvious clustering by diet was noted. Fig. 3b shows a volcano plot of the identified bovine, barley and microbial proteins on the two diets, although 6 proteins, including bovine outer dense fibre protein 2, barley hexosyltransferase and IPPc domain-containing protein were not shown in the volcano plot having extremely low *p*-values. In total, 39 of 846 proteins were differentially abundant ($p < 0.05$ and ATB/PTB > 1.5 or PTB/ATB > 1.5) between diets, 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 (Table 1). Based on gene-enrichment and functional annotation analysis, 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 (Fig. 4a). Proteins that are involved in catabolism of lipid and development of digestive tract were also identified.

Among the top 20 bovine proteins (Fig. 2a) identified with abundance compared between groups there were: glycoproteins (mucin-2 and glycoprotein 2), albumin, mucosal pentraxin 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 infection and inflammation including IgG-FC binding protein, complement C3 and polymeric immunoglobulin receptor. Examination of bovine DAP (Table 1) showed there were 12 proteins that were significantly lower ($p < 0.05$) in ATB than PTB but only Outer dense fibre protein 2, 2′-5′-oligoadenylate synthase-like, and DNA helicase were significantly lower in ATB than PTB on the basis of adjusted *p*-value <0.05 with 4 proteins increased on p-value but no protein being significantly higher with adjusted $p < 0.05$. Relative abundances assessed between protein by Mascot analysis (Table 2) revealed that albumin had the highest abundance index with emPAI of 6.95 followed by mucosal pentraxin (6.30), lysozyme C (2.98) and phospholipase A2 (2.91). In Table 2, while 73 bovine proteins were identified by Mascot, the proteins listed are those with Mascot Score > 99 and with >1 unique peptide matched. Differing criteria were used for bovine, barley, clostridium and methanobrevibacter proteins in Table 2 in order to filter for proteins to include those based on total numbers of proteins per genera and number of unique peptides identified.

One hundred and ninety-nine barley proteins were identified in the faeces (Fig. 2b), of which, in the TMT analysis between groups serpin Z4 was the most abundant across dietary groups and actin, elongation factor Tu and elongation factor 1-alpha were also among the top 20 abundant proteins in the comparison between groups. Barley enzymes were also found in the faeces, including telomerase reverse transcriptase, E1 ubiquitin-activating enzyme, peroxidase, hexosyltransferase, E3 ubiquitin ligase, acetyl-CoA carboxylase and 1,3-beta-glucan synthase. For barley (Table 1), there were 11 proteins that were significantly lower ($p < 0.05$) in ATB than PTB but only Hexosyltransferase, IPPc domain-containing protein and three uncharacterised proteins were significantly lower in ATB than PTB on the basis of adjusted $p < 0.05$. Relative abundances assessed between protein by Mascot analysis (Table 2) revealed that serpin Z4 had the highest abundance index emPAI of 1.66 followed by alpha-amylase inhibitor BDAI-1 (0.73), glyceraldehyde-3-phosphae dehydrogenase (0.53) and serpin Z7 (0.39). For Table 2, while 22 barley proteins were identified by Mascot, the proteins listed are all proteins with >1 unique peptide.

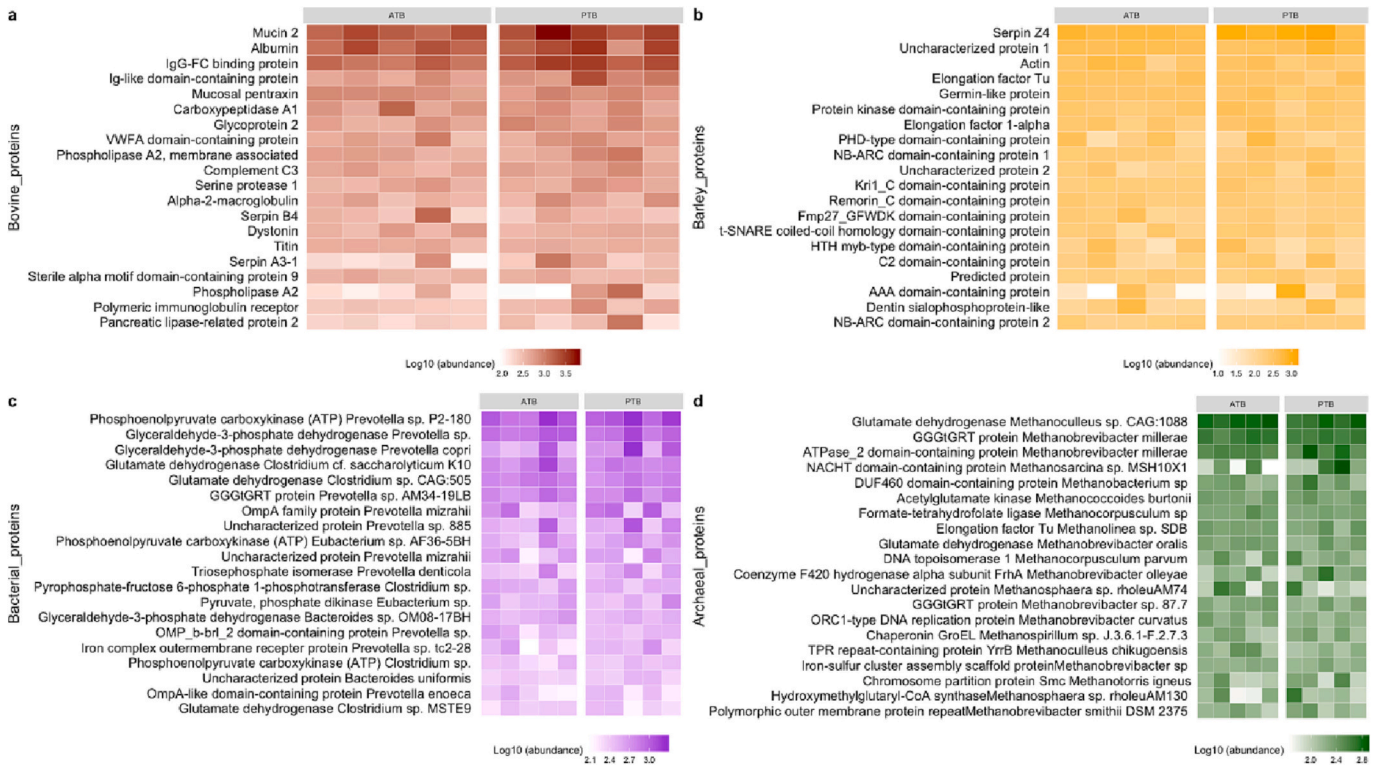

Fig. 2. The 20 most abundant (a) host, (b) barley, (c) bacterial and (d) archaeal proteins in the bovine faeces. The heatmaps show the relative abundance between the ATB and PTB groups where $n = 5$.

In total, 176 bacterial proteins were identified from genera *Clostridium* (91), *Prevotella* (46), *Bacteroides* (17), *Ruminococcus* (14) and *Eubacterium* (8). One hundred and ninety proteins of archaeal methanogenic origin 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 significantly modulated bacterial and archaeal proteins in the TMT study comparing the dietary groups in the bovine faeces are shown in Fig. 2c and Fig. 2d, respectively. The microbial proteins in the bovine faeces were over-represented in biological processes including carbohydrate metabolism, gluconeogenesis, glycolysis and glucose metabolism. Fig. 4b shows the biological processes in which the most faecal microbial proteins were involved (at least 10 unique peptides were matched per process). In bacteria though 2 proteins were significantly lower ($p < 0.05$) in ATB than PTB (Table 1), only chaperonin GroEL was significantly lower in ATB than PTB on adjusted $p < 0.05$. Two proteins were significantly higher in ATB than PTB with $p < 0.05$ but none were significant on adjusted p-value. In Archaea, there were 4 proteins significantly lower in ATB than PTB on the basis of $p < 0.05$, but only putative ABC transport system ATP-binding protein was significantly lower in ATB than PTB on adjusted $p < 0.05$ and one protein was significantly higher in ATB compared to PTB groups at $p < 0.05$ but was not significant at adjusted $p < 0.05$. Relative abundances among bacterial proteins assessed between protein by Mascot analysis (Table 2) revealed that *Clostridium* type I glyceraldehyde-3-phosphate dehydrogenase had the highest abundance index emPAI of 0.90 followed by phosphoenolpyruvate carboxykinase (0.88) and nifU-related domain containing protein (0.74). In archaea *Methanobrevibacter* GGGtGRT protein (0.68) and nitrogen-fixing protein NifU (0.45) had the highest emPAI. For Table 2, 116 *Clostridium* proteins were identified by Mascot with those listed having Mascot score > 99 and 1 or more unique peptide with multiple isoforms eliminated, while 18 *Methanobrevibacter* proteins were reported on Mascot and those listed in Table 2 had Mascot score > 99 and 1 or more unique peptides.

3.2. Validation of proteomics

The presence of serum albumin and serpin Z4 in bovine faeces was verified by WB (Fig. 5a and Fig. 5b; full images are shown in Supplementary Fig. S1), which were quantified by Image J densitometry and compared to proteomic abundance (Figs. 5c-f). The positive controls for albumin detection on the WB (purified bovine serum albumin) and for serpin Z4 (barley 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 of both ATB and PTB groups. However, there were no significantly differences between 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) (Fig. 5c-f).

4. Discussion

The present study identified host, dietary and microbial proteins in bovine faeces. Bovine proteins and glycoproteins were present in the faeces many of which were host digestive enzymes, as well as a number of endogenous 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, methanogenic proteins from *Methanobrevibacter*, *Methanosarcina* and *Methanobacterium* were present.

There were relatively few DAP (Table 1) with statistically significant differences between the ATB and PTB groups. Most of the DAP with significant differences were more abundant in PTB than those in ATB. Host proteins with changed abundances were related to relevant

Table 1
Differentially abundant proteins between ATB and PTB groups as determined by TMT labelled analysis, listed in order of Log_2 (ATB/PTB).

No	Protein	Accession	Log_2(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

biological processes such as endopeptidase activity, responses to Gram-negative bacteria, while bacterial proteome modifications were also relevant to the GIT microbiome such translation, gluconeogenesis and carbohydrate metabolism. Indeed starch degradation was suggested previously to be specific for *Bacteroidetes* and was one of the most relevant metabolic pathways of sheep faecal microbiome [20]. However in this study, identified proteins related to starch utilization system such as TonB-dependent receptors from *Bacteroidetes* and *Prevotella,* were not found to differ between groups. The diversity of bacterial community was found to be significantly decreased as digesta passed through the GIT [21], and the diet-induced changes in the rumen bacteria were reduced or eliminated in the faeces [22], which might explain the identifications of few differentially abundant proteins between groups. The adaptation of animals to the diets, the small sample size, the integrity of the database and the large variation of protein abundances within the groups of cattle fed with the ATB and PTB diets contributed to the low number of DAP. However, the study has provided a valuable insight on the varied contributions to the bovine faecal proteome from the host, feed and microbial population which warrants further consideration regarding their presence in faeces and their relative abundance as estimated by their emPAI.

4.1. Bovine proteins

Faeces constantly sample the cellular environment when passing down the GIT. Two hundred and eighty-one host proteins were quantified in the TMT labelling analysis approach in the present study, including proteins likely 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) [7], possibly because the bovine database was smaller than that of human. Relatively small numbers of host proteins were also reported in faeces of mice (115 proteins) [9] and sheep (431 proteins) [10]. Consistent with a previous study on sheep [10], serum albumin, IgG-FC binding protein, mucin 2, serine protease 1, polymeric immunoglobulin receptor (PIGR) and mucosal pentraxin were present in the bovine faeces as had been reported for ovine faeces. Serum albumin in GIT has been usually reported to be associated with protein-losing enteropathy [23]. Despite the absence of indicators of clinical disease, the cattle in the present study were consuming high grain diets, and would be expected to have some degree of alteration to the gut as a consequence [24], explaining the presence of albumin in the bovine faecal extracts. Although high MW (> 190 kDa) and abundant glycoproteins were largely removed by using the IGSP method [14], glycoproteins including mucin 2 and glycoprotein 2 were still demonstrated in the present study. Mucin 2, mucin 5 AC 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; besides providing lubrication, they, especially the major intestinal mucin 2, are able to protect GIT from bacteria and self-digestion by resisting endogenous proteases [25]. Glycoprotein 2 is secreted by the pancreas and is present in the outer mucin layer of

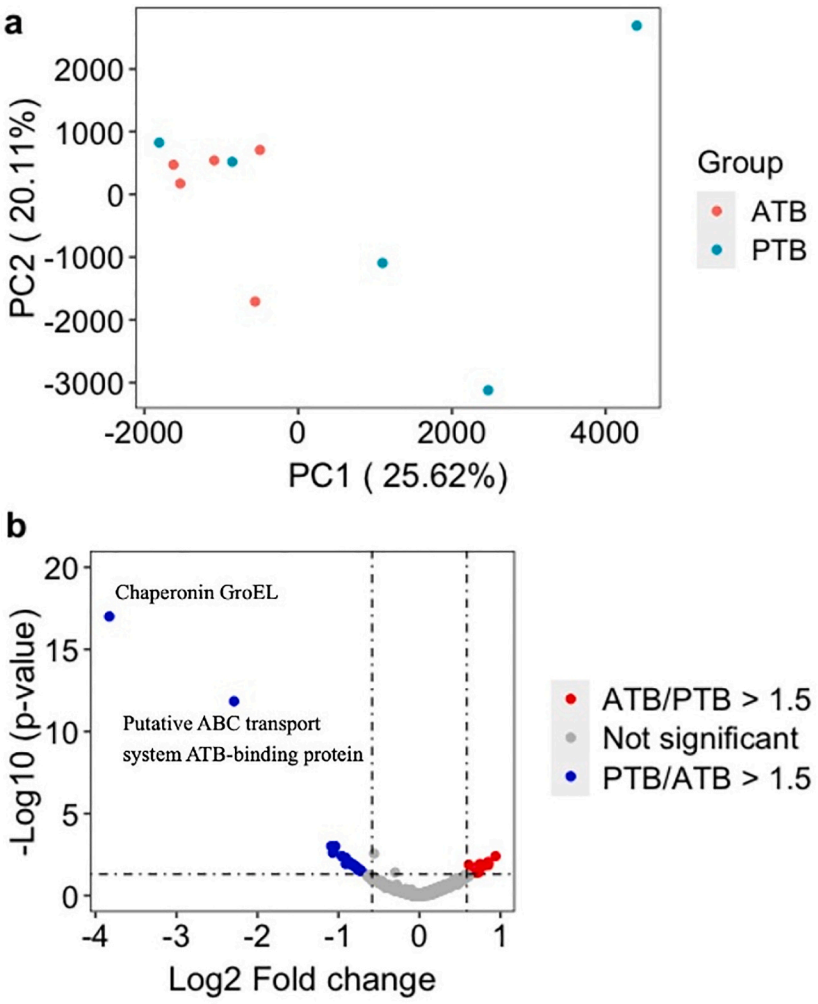

Fig. 3. (a) Scatter plot of the PCA of the bovine faecal samples, and (b) volcano plot of all the identified faecal bovine, barley and microbial proteins. In (b), the two vertical dotted lines represent fold change of ATB and PTB equal to 1.5 or 2/3, and the horizontal dotted line represents *p*-value 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. Six proteins, including bovine outer dense fibre protein 2 and barley hexosyltransferase and IPPc domain-containing protein were not shown in the volcano plot having extremely low *p*-values. Chaperonin GroEL is a protein in *Clostridium*, while putative ABC transport system ATB-binding protein is from *Methanococcus*.

the colon. It has been found to be co-localized with mucin 2, playing an important role in defence against bacteria during intestinal inflammation [26]. Among the proteins which were also involved in inflammation, PIGR, complement C3 and immunoglobulin J chain were identified in the bovine faeces and have also been found in monkey faeces [3]. Mucosal pentraxin was identified 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 [27]. It 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 [27]. The observation of these proteins in the bovine faeces by proteomics may suggest novel means to characterise the intestinal health of animals.

Many host enzymes related to intestinal digestion were identified in the present study, including carboxypeptidase A1, serine protease 1, phospholipase A2, membrane associated phospholipase A2 and pancreatic lipase-related protein 2, which were among the top 20 most abundant host proteins. Carboxypeptidase A1 is a zinc-dependent exopeptidase secreted from pancreas and activated by trypsin in the duodenum [28]. It acts on smaller polypeptides after their initial breakdown by endopeptidases, leading to the formation of amino acids [28] for absorption in the small intestine. However, it was also found to be 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 [2], being explained by an increase rate in passage and/or reduction of proteolytic degradation [29]. Aminopeptidase N, mainly located in small-intestinal or renal microvillar membrane, was also found in the bovine faeces. It is important in the final digestion of peptides, and has been reported to be involved in cell motility and adhesion [30]. Alpha-amylase and maltase-glucoamylase, which work synergistically in digesting starch in small intestine, were also identified in the faeces. Although there was a significant difference in faecal starch concentration between groups (unpublished data), no differences were found in these enzymes. However, differences in faecal starch may be due to a difference in the availability of the starch for enzymic action, caused by the ammonia or sodium propionate treatments. Alpha-2-macroglobulin, a broad-spectrum protease inhibitor, was abundant in the bovine faeces. It inhibits serine-, cysteine- and metalloproteinases, and plays an important role in inflammation, immunity and infection [31]. Serpin A3–1 and serpin A3–7 were also found in the bovine faeces. Serpin A3–1 selectively inhibited trypsin but not chymotrypsin, elastase or subtilisin, while serpin A3–7 selectively inhibited papain-like cysteine and elastase-like serine proteases, but not chymotrypsin, trypsin, plasmin, thrombin, furin or cathepsin B [32]. 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 below, showed a relatively high abundance of protease inhibitors in the bovine faeces.

In terms of the relative abundance in comparing between the bovine proteins using the Mascot search engine, mucosal pentraxin and albumin had the highest emPAI, not only for bovine proteins but they were also the most abundant proteins across all taxonomies assessed. The presence

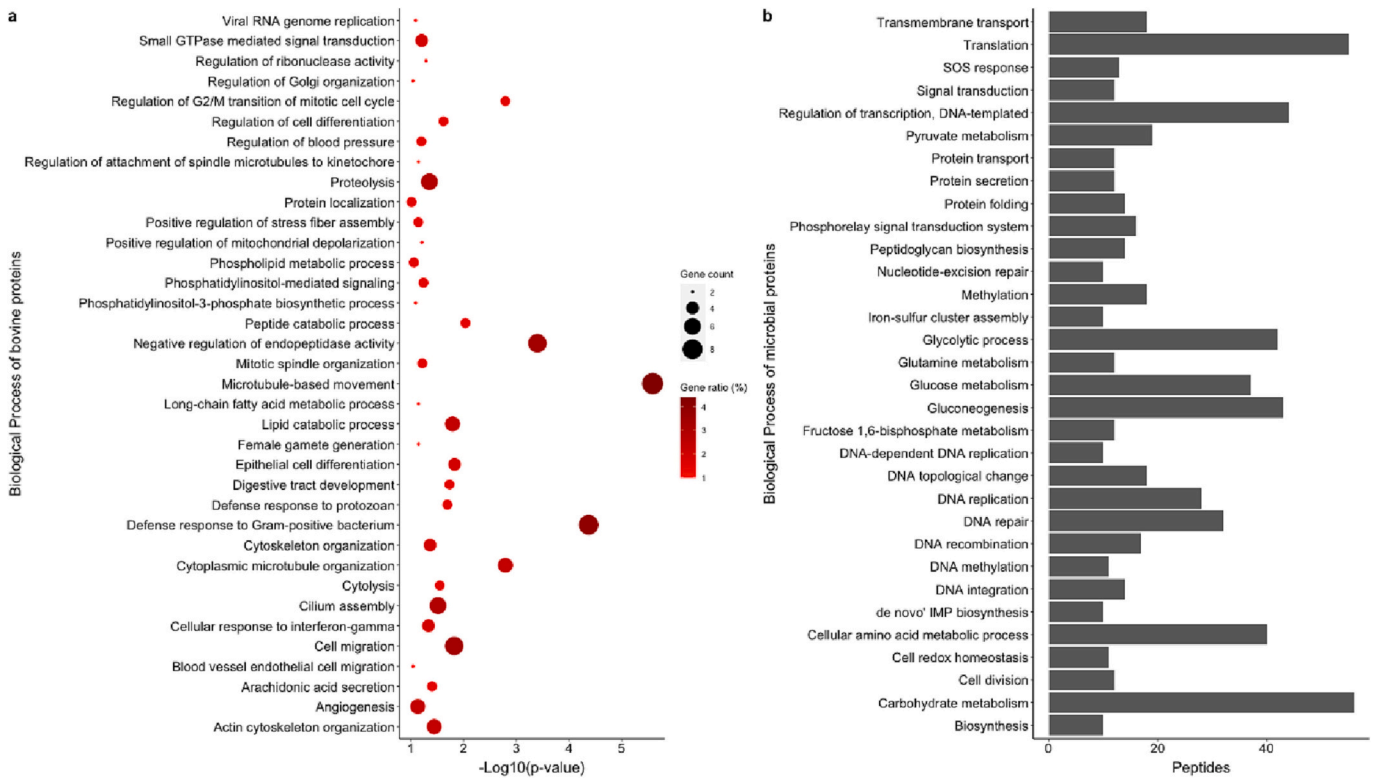

Fig. 4. (a) Gene-enrichment and functional annotation analysis of the host proteins, and (b) the biological processes in which most faecal microbial proteins were involved. *P*-values in (a) are EASE scores in DAVID, which are modified Fisher exact *p*-values; gene ratio (%) represents the percentage of the identified genes in the total genes of the given process.

of albumin in the faecal extracts was validated by western blot (Fig. 5a) which showed that 7 of the 10 samples used in the study had distinct bands cross reacting with the specific ant-bovine serum albumin antibody. No antibody was available to validate the finding of a high abundance of mucosal pentraxin but the TMT analysis (Fig. 2) does indicate it is a present at around the same abundance in each sample. The high abundance of this intestinal protein in bovine faeces warrants further investigation especially with its similarity to other mammalian pentraxins such as C-reactive protein and serum amyloid P [27].

4.2. Barley serpin Z4 and Z7

Barley serpin Z4 and serpin Z7 were identified in bovine faeces by TMT proteomics and the presence of serpin Z4 was confirmed by WB, although serpin Z7, the upper band showing in the WB of barley extract [16] (track 1, Fig. 5b), 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. These serine protease inhibitors survive through malting and fermentation in the beer brewing process [33]. Serpin Z4 was found to be positively correlated with beer foam that forms the head on poured beer and has been identified as a marker of foam stability [34], while correlation between serpin Z7 and beer foam was negative [35]. The persistence through the digestive process of dietary proteins that are involved with foam stability might have implications for foam formation in frothy bloat of cattle, a severe disease in this species that can be fatal [36] and would be worthy of future research to address this observation.

The protease inhibition by serpins and some other plant-derived protease inhibitors in the intestinal tract have been reported [37–39] 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 alternatively over-secretion of the digestive enzymes [37,40]. 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 reducing the availability of amino acids for their growth and development [38]; serpin-1 in pumpkin was negatively correlated with aphid survival, suggesting a potential role for plant serpins as insect inhibitors [39]. However, there is no study to date investigating the action of serpin Z4 or Z7 in inhibition of mammalian digestive enzymes. If barley serpin Z4 and Z7 impair digestion by inhibiting proteases, knocking out their genes or inhibiting their expression in barley might be expected to enhance the absorption of nutrients in animals. Finally, some plant-derived protease inhibitors have also been reported to play an important role in inflammatory responses in the GIT, mitigating inflammation and gastric pain [37] and to have anticarcinogenic properties [41]. The detection and quantification of serpin Z4 and serpin Z7 in the faeces may provide a new research direction for ruminant digestion and absorption, and diseases.

The emPAI data confirmed that serpin Z4 was the most abundant barley protein in the bovine faecal extracts having survived the GIT process which include fermentation in the rumen and the action of endogenous proteases in the intestine. Most of the other barley proteins with abundance determined by emPAI, were also protease inhibitors and summation of both barley anti-proteases: serpin-Z4, serpin-Z7, alpha-amylase/trypsin inhibitor with the endogenous bovine anti-proteases: serpin A3–2, alpha-1-antiproteinase, alpha-2-macroglobulin gave a total emPAI of 3.99. Of interest is that summation of the bovine proteases in the faecal extract: carboxypeptidase A1, serine protease 1, chymotrypsin-like elastase family member 1, chymotrypsin-C gave an emPAI of 4.52. While the similarity of these summed emPAI may be coincidental it could relate to the mechanism of action of the anti-proteases in which serpins bind to proteases in a 1:1 stoichiometry [37]. Elucidation of the interaction of protease and anti-protease in the bovine GIT would be worthy of further examination.

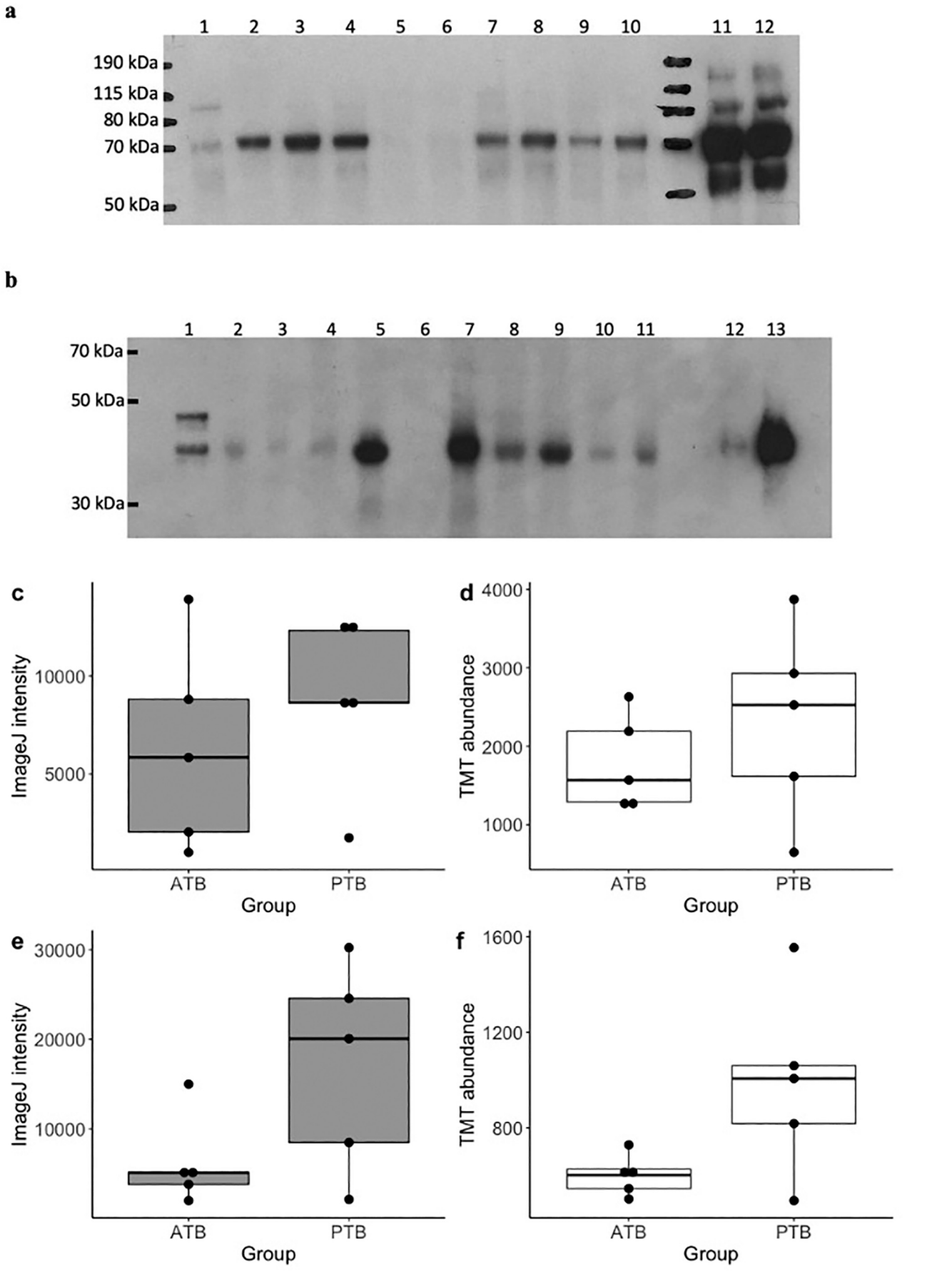

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Fig. 5. Western blot (WB) analysis of (a) bovine serum albumin and (b) barley serpin Z4 and relative amount of albumin and serpin Z4 determined by ImageJ analysis of WB band intensity (c and e) and TMT-based proteomic analysis (d and f). Faecal samples 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 Best, Belhaven Brewery, Belhaven, UK).

Table 2
Mascot search results on the pooled sample after TMT labelling, listed by emPAI.

Protein	Uniprot Number	Mascot Score	Peptides Unique (total)	emPAI
Bovine				
Mucosal pentraxin	Q3T166	404	11 (11)	6.95
Albumin	P02769	824	27 (27)	6.3
Lysozyme C	PO4421	117	5 (5)	2.98
Phospholipase A2, membrane associated	Q56JZ2	108	4 (4)	2.91
Carboxypeptidase A1	P00730	369	12 (13)	2.66
Serine protease 1	P00760	180	4 (4)	1.03
Annexin A4	P13214	198	5 (5)	0.92
Chymotrypsin-like elastase family member 1	Q28153	146	3 (3)	0.63
Chymotrypsin-C	Q7M3E	117	3 (3)	0.6
Alpha-1-antiproteinase	P34955	146	4 (4)	0.5
Serpin A3–2	A217M	115	4 (5)	0.5
Complement C3	Q2UVX4	260	12 (12)	0.39
Alpha-2-macroglobulin	Q7SIH1	219	7 (7)	0.22
Keratin, type II cytoskeletal 7	Q29S21	100	2 (2)	0.2
Polymeric immunoglobulin receptor	P81265	104	3 (3)	0.18
Barley				
Serpin-Z4	P06293	457	11	1.66
Alpha-amylase inhibitor BDAI-1	P13691	21	2	0.73
Alpha-amylase/trypsin inhibitor	P32936	57	2	0.72
Glyceraldehyde-3-phosphate dehydrogenase	P08477	38	3	0.53
Serpin-Z7	Q43492	135	3	0.39
Bacteria				
Clostridium				
Type I glyceraldehyde-3-phosphate dehydrogenase	WP 066547095.1	150	2 (5)	0.90
Phosphoenolpyruvate carboxykinase	SCH76716.1	339	3 (7)*	0.88
nifU-related domain containing protein	CDC09873.1	221	1 (2)	0.74
Hypothetical protein CLOACE_04350	0FI0243.1	221	1 (2)	0.58
GGGtGRT protein	WP 022001700.1	130	1 (3)	0.48
Ketol-acid reductoisomerase	WP 090015105.1	168	1 (3)	0.46
Pyruvate phosphate dikinase	CDC60454.1	153	1 (4)	0.27
Formate–tetrahydrofolate ligase	WP195379501.1	164	1 (3)	0.26
Glutamate dehydrogenase	WP 139905928.1	119	1 (2)	0.26
Archaea				
Methanobrevibacter				
GGGtGRT protein	WP042707039.1	158	4 (4)	0.68
NADP-specific glutamate dehydrogenase	WP069575012.1	132	4 (4)	0.47
Nitrogen-fixing protein NifU	WP012954993.1	263	1 (1)	0.45

4.3. Microbial proteins

In the present study, bacterial proteins from *Clostridium* were predominant, followed by *Prevotella*, *Bacteroides*, *Ruminococcus* and *Eubacterium*. This was similar to findings in previous genomic studies [42], which showed that *Clostridium* was the most abundant bacterial genus in bovine faeces, and reported the same gene enrichment order of the five genera as our findings except that *Bacteroides* were more abundant than *Prevotella*. Genera *Prevotella* and *Ruminococcus* had been considered core bacteria as they were present in most studied bovine faeces as well as rumen samples [4]. *Clostridium* and *Bacteroides* were abundant in the rectum [43,44], and they were enriched in the cecum and colon of calves compared to the rumen and were increased in the cecum as calf grew [45]. *Methanobrevibacter* is the most representative archaea in ruminants and commonly present in the gut of ruminants [46]. Many methanogenic archaeal proteins were identified in the present study, among them, *Methanobrevibacter* was the dominant genus, followed by *Methanosarcina* and *Methanobacterium*.

Bacterial metabolism can produce short-chain fatty acids from plant polysaccharides in the GIT, contributing significantly to host energy balance. The identified faecal microbiome was over-represented in carbohydrate metabolism; translation; regulation of DNA-templated transcription; gluconeogenesis; glycolysis; glucose metabolism and cellular amino acid metabolism. Our findings were consistent with a multi-omics study on sheep faeces [20], which demonstrated that the sheep faecal microbiome was primarily involved in catabolism, and peptidoglycan biosynthesis, glycolysis and gluconeogenesis were the three most relevant metabolic pathways based on protein abundance; and Mao et al. [44] had also found that a high proportion of the bovine faecal bacterial genes was related to carbohydrate and protein metabolism. Ruminants contribute to a large proportion of methane emissions [47]. Methane can be synthesized by methanogens using H_2, CO_2, formate, methyl compounds or acetate as substrates [48]. Methane emission can be predicted from dietary input variables such as dry matter intake, digestibility of hemicellulose, and metabolizable energy intake [49]. Many dietary strategies, including using secondary plant metabolites (e.g., tannins and saponins) [50], seaweed and 3-nitrooxypropanol [51], have been proposed for methane mitigation in ruminants. Some researchers also found that animals which had lower residual feed intake could produce less methane [52]. 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.

The examination of emPAI for relative abundance estimation for bacterial and archaeal proteins was limited to clostridium and methanobrevibacter taxonomies which were those with the most prominent proteins in the TMT based proteomics. The emPAI did reveal that these proteins had abundancies in the emPAI range of 0.26–0.9 with none of the proteins at the abundances seen with bovine or barley protein,.

4.4. Limitations of the study

A limitation of the study was the small sample size which related to it being an initial study with limited resource. Though the in overall study over 200 steers had been fed with either ATB or PTB and ideally the group sizes could have been increased to provide greater statistical power it was not possible on this occasion, but the outcome obtained, revealed the value of using the relative quantitation provided by TMT-proteomics justify this experimental approach further justified by the

novel findings on survival of barley protein through the GIT . Another limitation of the study was that the microbial proteins were searched in databases of pre-selected genera, which are abundant or common in the ruminant GIT having been reported by earlier studies [4,20]. This was done to lower computational load, limit the analysis time for the search and prevent an increase in false discovery rates, and while useful in this initial investigation of bovine faecal extracts, in future investigations more sample replicates would be included and a metagenome analysis would be initially performed to ensure that all relevant species would be included in the proteomic analysis. Furthermore, investigation using absolute quantification by methods such as DIA, SWATH-MS or spectral counting rather than the relative quantification as used here, would be valuable to confirm these initial findings on the proteins of bovine faeces. Further studies are also needed to have a deeper understanding of the effect of diet on faeces, but the ability to concurrently identify and quantify proteins from the host, the feed and from bacteria or archaea in bovine faeces as in this investigation provide a foundation for fruitful future research.

5. Conclusions

The present study characterised a bovine faecal proteome of 281 bovine, 199 barley, 176 bacterial and 190 archaeal proteins. Mucosal pentraxin and albumin were the most abundant host proteins identified in the faeces, while many host digestive enzymes and protease inhibitors were also found. Barley serpin Z4, a serine protease inhibitor, 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*, while *Methanobrevibacter* was the dominant archaeal genus. The majority of DAP between groups were more abundant in the PTB group compared to the ATB group. A key finding of the study was demonstration that protein from barley in the feed of the cows could survive the ruminal fermentation and intestinal digestive processes of the GIT, while recognition that barley protease inhibitors are the most abundant proteins from feed to survive the tract, stimulates a range of possible future research directions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2023.104941.

Funding

Harbro Limited provided funding for the initial cattle study and the proteomic evaluations. The University of Glasgow and China Scholarship Council supported the studentship.

CRediT authorship contribution statement

Y. Huang: Methodology, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **N.N. Jonsson:** Conceptualization, Formal analysis, Visualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing. **M. McLaughlin:** Validation, Writing – original draft, Writing – review & editing. **R. Burchmore:** Methodology, Writing – original draft, Writing – review & editing. **P.C.D. Johnson:** Formal analysis, Visualization, Supervision, Writing – original draft, Writing – review & editing. **R. Jones:** Validation, Writing – original draft, Writing – review & editing. **S. McGill:** Methodology, Writing – original draft, Writing – review & editing. **N. Brady:** Validation, Writing – original draft, Writing – review & editing. **S. Weidt:** Methodology, Writing – original draft, Writing – review & editing. **P.D. Eckersall:** Conceptualization, Methodology, Formal analysis, Visualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

The mass spectrometry proteomics data supporting the conclusions of this article are available in the ProteomeXchange Consortium, PXD036027. Supplementary information can be found in the additional file.

Acknowledgements

We thank the team of Professor Greg Tanner from the University of Melbourne for the barley serpin Z4 antibody. Yixin Huang thanks the China Scholarship Council and the University of Glasgow for the support for her PhD study.

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