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Functional metabolic capacity of pig colonocytes is differentially modulated by fermentable fibre and poorly digestible protein

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ABSTRACT

The intestine is a highly metabolic organ that relies on energy production within the intestinal cells to sustain its functions. In the colon, intestinal cell metabolic function could be affected positively and negatively by microbiota-derived metabolites. Protein fermentation metabolites are known to negatively impact intestinal metabolic function, while fibre fermentation metabolites are generally thought beneficial. We aimed to investigate whether proteins of different digestibility in the absence and presence of fibres impact the energy metabolism of colonocytes, with potentially adverse health effects. We fed 32, 9-week-old boars one of four experimental diets for 14 days in a 2 × 2 factorial arrangement. Whey and collagen were added as a well and a poorly digestible protein source, respectively, and fibre was either included at 5% or 23%. We examined the effects of the diets on the flux of fermentation metabolites in colon digesta and assessed the impact of the diets on functional metabolic capacity of isolated colonocytes using the Seahorse XF analyzer. Feeding the poorly digestible protein source collagen indeed increased nitrogen flow into the colon by 135% compared to the well-digestible whey-protein source. Feeding high fermentable fibre increased colonic fluxes of both fibre-derived metabolites acetate, propionate, butyrate and caproate, but also increased flux of protein-derived metabolites ammonia, isobutyrate, isovalerate, valerate and isocaproate. To analyse the impact of the diets and the induced differential metabolic composition of the intestinal lumen on functional metabolic capacity of the intestine, we used extracellular flux analysis on freshly isolated pig colonocytes. Colonocytes isolated from high fermentable fibre-fed pigs in the whey-protein diet, but not in the collagen-protein diet, had a reduced mitochondrial capacity, as indicated by a 35% reduction of maximal respiration (interaction $P < 0.05$) and a 20% reduction of spare respiratory capacity (interaction $P < 0.05$). Colonocytes from high fermentable fibre-fed pigs had a 37% decreased glycolytic activity compared to the colonocytes isolated from the low fermentable fibre-fed pigs ($P < 0.001$). This indicated that different diets, and in particular different protein sources and fibre levels, differentially affect colonic epithelial cell metabolism in pigs. Especially, high fermentable fibre lowered both colonocyte mitochondrial and glycolytic metabolism, indicating that high-fibre intake in pigs could lower colonocyte energetic status. Because the metabolic capacity of colonocytes is tightly linked with their functionality, assessment of intestinal cell metabolic capacity may be a valuable tool for future research.

Implications

Sufficient energy production is important to support intestinal functions. In this study, we investigated whether dietary interventions, designed to impact colonic microbial fermentation, could alter the metabolic function of colonocytes. To do this, we isolated pig colonocytes and measured their metabolic capacity. We found that metabolic capacity of colonocytes was indeed modulated by two-week-long dietary interventions; protein source and fibre level differentially affected metabolic capacity. These results not only provide new insights into how diets impact intestinal health but also show that assessing colonocyte metabolic function may be a sensitive tool to investigate the effects of diets on the intestine.

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Introduction

The intestine is a major metabolic organ, which facilitates the selective entry of nutrients into the body, while simultaneously providing a barrier against pathogens. The intestine's absorptive and barrier functions are actualised by intestinal epithelial cells (**IECs**), a type of cell that is continuously generated from stem cells in the crypts, which migrate to the villus top as they mature, in a 5–7 day cycle (Potten et al., 1997). IECs form a physical barrier to protect the host, comprising a tight layer with actively maintained cell–cell junctions and specialised cells that secrete mucus to create a protective layer on top of the IECs (Peterson and Artis, 2014). To perform its complex role, the intestine is divided into various regions with distinct functions, which comprise an enormous surface area, resulting in a large, dynamic and active tissue with high energy demand. The specific energy demand for the intestinal processing of nutrients is illustrated by a 25% increase in resting metabolic rate for humans (Rolfe and Brown, 1997), and 15–20% for growing pigs (van Erp et al., 2018). This increase reflects a doubling of intestinal energy use, which is required to support digestion, absorption and metabolism of sugars, proteins and lipids. In addition, adequate cellular energy production was shown to be important for barrier integrity, and thus to maintain intestinal health (Rodenburg et al., 2008; Peng et al., 2009; Miao et al., 2016). Disruption of intestinal energy production, for example due to the absence of energy substrates or because of drug use, was found to increase intestinal apoptosis and permeability (Bjarnason and Takeuchi, 2009; Donohoe et al., 2011; JanssenDuijghuijsen et al., 2017). As such, intestinal energy metabolism emerges as an area of interest for the overall health of organisms.

For their metabolism, IECs use dietary energy substrates, of which the availability differs across the intestinal tract. Rapidly digestible carbohydrates and proteins are metabolised and absorbed in the small intestine (Drochner, 1993). Complex carbohydrates like fibres serve as substrates for microbiota, mainly in the colon (Drochner, 1993; Gilbert et al., 2018). Microbial fibre fermentation results in, among others, the production of short-chain fatty acids (**SCFAs**). One of the SCFAs, butyrate, is considered a preferred energy substrate of colonocytes (Roediger, 1980). Similar to carbohydrates, undigested proteins are subject to fermentation in the colon. Whereas colonic proteins can be used for microbial protein synthesis, they can also be used as microbial energy sources, leading to the production of a wide variety of protein fermentation metabolites (Pieper et al., 2016). Protein fermentation metabolites are thought to contribute to intestinal dysfunction by negatively impacting intestinal epithelial cell function, such as by disrupting colonocyte metabolic function (Blachier et al., 2007; Gilbert et al., 2018). A good example of a harmful protein fermentation metabolite is ammonia. Ammonia was shown to decrease the ability of colonocytes to oxidise butyrate, their primary energy source (Roediger, 1980; Darcy-Vrillon et al., 1996). In this study, we addressed whether functional colonocyte metabolism is challenged by protein fermentation metabolites. Most studies have not measured colonocyte energy metabolism directly, but we are able to do this with a recently optimised protocol to assess the energy metabolism of isolated IECs, measuring their oxidative and glycolytic fluxes (Bekebrede et al., 2021). This allows us to study the effects of feeds on colonic energy metabolism.

Given the upcoming feed transition to increased use of high-fibre co-products that are also rich in moderately digestible proteins (Zijlstra and Beltranena, 2019), we were interested in better understanding the interactions between protein and fibre in the colon. In general, adding fermentable fibre into pig feeds is thought to improve animal health through multiple mechanisms (Bikker et al., 2006; Jha and Berrocoso, 2016). Firstly, it provides butyrate to colonocytes. Secondly, it could reduce the production of metabolites originating from the fermentation of undigested proteins (Jeaurond et al., 2008; Kim et al., 2008). Thirdly, fibre fermentation metabolites, in particular butyrate, are known for their beneficial effects, such as improving barrier function (Peng et al., 2009), and acting as an anti-inflammatory (Schulthess et al., 2019) and anti-tumorigenic agents (Donohoe et al., 2012; Li et al., 2018).

In the current study, we investigated whether proteins of different digestibility in the absence and presence of fibres impact colonic energy metabolism, thereby potentially affecting pig health. To investigate this, we fed pigs diets in a 2 × 2 factorial arrangement, with protein source and fibre level as factors. Protein sources were either highly (whey) or poorly digestible (collagen), and for each protein source, fibre was included at either 5% or 23%, where starch was exchanged for soybean- and oat hulls in the high-fibre diets. We examined the effects of the diets on colonic fermentation metabolites and assessed the impact of the diets on functional metabolic capacity of IECs.

Material and methods

Animals

A total of 32 boars, 9 weeks of age, 22.8 kg BW (SEM = 0.5) (TN70; Large White × Norwegian Landrace, TopigsNorsvin, Vught, The Netherlands) originating from a specific pathogen-free breeding farm were transported to CARUS, the research facility of Wageningen University, the Netherlands. Pigs were group-housed with four pigs per pen, in 5 m^2 partly slatted pens with thick rubber mats for lying comfort. Upon arrival, pigs were weighed and allocated to the pens based on their initial BW, to minimise variation in BW between pens and treatments. Temperature was kept between 22 and 25 °C with a humidity of 65%, and lights and radio were switched on between 0700 and 1900 h and lights were dimmed to 5%, and radio was off from 1900 till 0700 h. The first two days, pigs were gradually switched to the experimental diets, which they received throughout the next two weeks. Health and welfare were assessed visually twice a day during feeding, and the faecal consistency score was assessed (score of 1–5, 1 = liquid diarrhoea, 5 = hard faeces).

The feeds were produced in meal form by Research Diet Services (Wijk bij Duurstede, The Netherlands) in a single batch. Collagen protein and the amino acids L-Phenylalanine and L-Leucine were mixed into the feeds at the research facility. Diets contained different protein sources and fibre levels in a 2 × 2 factorial design (Table 1). The experimental diets contained either whey as a highly digestible protein source or collagen as a poorly digestible protein source. For each protein source, there was a low-fibre diet that contained only 5% dietary fibre, and a high-fibre diet that contained 23% dietary fibre. To create the high-fibre diets, maize starch was exchanged for soybean hulls and oat hulls. Synthetic amino acids were added to the collagen diets to meet the minimal requirements for essential amino acids. Pigs were fed twice a day at 0800 h and 1600 h, and received 50% of the daily feed allowance per meal. Feed intake was set at 1.4 times the maintenance energy requirement using the formula from CVB (net energy for maintenance = 750 × $BW^{0.60}$, (Everts, 2015)), to ensure that all pigs consumed the same amount of feed. To determine nutrient digestibility and the mean intestinal retention time of the diets, TiO_2 was included at 2 g/kg as an indigestible marker. Based on previous studies, it was modelled that feeding pigs every 6 hours for at least 36 h could provide a constant flow of digesta through the colon (de Vries and Gerrits, 2018; Martens et al., 2019; Schop, 2020). Therefore, starting 36 hours before slaughter, pigs were fed every 6 hours with 25% of the daily feed allowance, and

Table 1
Ingredient and calculated nutrient composition of the four experimental diets fed to pigs for two weeks, expressed on an as-fed basis.[1]

	Whey		Collagen	
Components	Low Fibre	High Fibre	Low Fibre	High Fibre
Ingredient composition (%)				
Whey Protein isolate	21	21	–	–
Bovine Collagen	–	–	18.1	18.1
Soy Protein Concentrate	3.1	0	3.1	0
L-Lysine HCl	0.05	–	0.64	0.58
DL-Methionine	–	–	0.5	0.5
L-Threonine	–	–	0.4	0.4
L-Tryptophan	–	–	0.2	0.2
L-Isoleucine	–	–	0.3	0.3
L-Histidine	–	–	0.3	0.3
L-Phenylalanine	–	–	0.3	0.3
L-Leucine	–	–	0.6	0.6
L-Valine	–	–	0.3	0.3
Sucrose	10	10	10	10
Wheat gluten meal	0.8	–	0.8	–
Maize starch	53.2	29	52.9	28.9
Soybean Hulls	–	15.5	–	15.5
Oat Hulls	–	13.2	–	13.2
Cellulose	3	3	3	3
Rape seed oil	3	3	3	3
Premix (vitamins + minerals)[2]	1	1	1	1
MgO	0.2	0.1	0.2	0.1
$CaCO_3$	1.5	1.2	1.5	1.2
$Ca(H_2PO_4)_2$	1.5	1.5	1.6	1.6
KCO_3	0.3	–	0.5	–
KCl	0.3	0.3	0.1	0.1
$NaHCO_3$	0.5	0.5	0.5	0.5
TiO_2	0.2	0.2	0.2	0.2
Total	100	100	100	100
Nutrient composition (g/kg)				
DM	920	921	924	925
CP	220	220	220	220
Digestible CP	194	179	177	162
Crude Fat	36	40	34	38
Crude Ash	40	49	42	49
Starch	513	303	510	302
Sugars	113	111	112	109
Dietary Fibre[3]	47.1	231.5	47	231.3
NE (MJ/kg)	11.6	9.5	11.4	9.4

Abbreviations: NE = net energy.
[1] Nutrient composition was calculated based on ingredient composition and table values for the composition of the ingredients (Centraal Veevoeder Bureau (CVB), 2018).
[2] Supplied per kilogram of feed: retinyl acetate, 10 000 IU; cholecalciferol, 2 000 IU; dl-a-tocopherol, 40 mg: menadione, 1.5 mg; thiamine 1.0 mg; riboflavin, 4 mg; pyridoxin-HCl, 1.5 mg; cyanocobalamin, 20 µg; niacin, 30 mg; D-pantothenic acid, 15 mg; Choline chloride, 150 mg; Folic acid, 0.4 mg; Biotin, 0.05 mg; iron(II)sulphate monohydrate, 331 mg; copper(II)sulphate pentahydrate, 80 mg; manganese(II)oxide, 49 mg; zinc sulphate monohydrate, 194 mg, potassium iodate, 1 mg; sodium selenite, 0.56 mg.
[3] Dietary fibre was calculated as follows: organic matter – CP – crude fat – starch – sugar.

constant flux of digesta through the colon was assumed for all diets in this study. We verified that each pig consumed its own portion of feed by visual observation. Water was available *ad libitum* throughout the entire study period. At the end of the study period, pigs were sedated using intramuscular injection of Zoletil + Xylazine (5:2 ratio, 1 mg/10 kg BW) and euthanised by lethal injection with pentobarbital (24 mg/kg BW).

Enterocyte isolations

Colonocytes were isolated according to (Bekebrede et al., 2021). Briefly, following excision from the abdominal cavity, the entire intestine was separated from the mesentery, and the colon was located. At 66% of the length of the colon, a clamp was placed to delineate the distal part of the colon. Then, a piece of 20 cm proximal of the clamp was removed and placed in aerated modified Krebs Henseleit Buffer (**KHB**) containing 5 mM glucose (#K3753, Sigma-Aldrich, hereafter referred to as modified KHB), supplemented with 2.5 g/L bovine serum albumin (**BSA**, #A7906, Sigma-Aldrich). To ensure that isolated cells maintained good viability, cell isolations for each slaughter day were performed simultaneously. Only eight pigs were slaughtered per day to ensure that each pig had at least eight replicates for the metabolic analysis, which is performed on a 96-well plate. When all samples were obtained, intestines were washed thoroughly with modified KHB and inverted. Using dialysis clamps (#Z371092, Sigma-Aldrich), a sac was created by filling the inverted intestine with modified KHB, which was then placed in 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 KH_2PO_4, 10 mM HEPES (Ca^{2+}-free KHB, pH 7.4), supplemented with 10 mM Dithiothreitol, 20 mM Ethylenediaminetetraacetic acid and 2.5 g/L fatty acid-free BSA (#3117057001, Sigma-Aldrich) to wash the mucus away. The intestines were incubated in this wash buffer for 20 minutes in an oscillating water bath at 37 °C. Then, the buffer was discarded, and the washing step was repeated for another 20 minutes, using the same Ca^{2+}-free KHB buffer supplemented with 10 mM Dithiothreitol, 20 mM Ethylenediaminetetraacetic acid and 2.5 g/L fatty acid-free BSA. The intestinal sacs were then reverted and filled with Ca^{2+}-free KHB buffer containing, 10 mM Dithiothreitol, 400 U/mL hyaluronidase type IV (#3884, Sigma-Aldrich) and 2.5 g/L fatty acid-free BSA. After a fifteen-minute incubation, the intestinal sacs were gently massaged for 15 seconds, after which the content

of the sacs was collected in 50 mL tubes. The cells were then passed through a 70 μM cellulose filter to remove debris and large tissue pieces. The collected cells were washed twice using modified KHB containing 2.5 g/L BSA and once with pH balanced XF DMEM assay medium supplemented with pH balanced 10 mM XF glucose, 2 mM XF glutamine and 1 mM XF pyruvate. Cells were spun down at 400*g* for 5 min. Then, cells were counted using the Bürker chamber, and cell viability was assessed by staining cells with ViaStain (#CS2-0106, Nexcelom Bioscience) and imaging them using the Cellometer K4 (Nexcelom Bioscience).

Metabolic measurements

The metabolic function of the isolated colonocytes was measured in real-time using the XFe96 Seahorse Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara, USA). Isolated colonocytes were plated into Seahorse 96-well plates at 100 000 cells/well. Because isolated colonocytes are non-adherent cells, the plates were precoated with CellTak (#354240, Corning, New York, USA) according to the manufacturer's protocol to ensure that cells adhere to the bottom of the plate throughout the measurement. After calibration and initialisation, basal metabolic function of the plated colonocytes was measured. Then, 1 μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazon (#C2920, Sigma-Aldrich), 2.5 μM Antimycin A (#A8674, Sigma-Aldrich) and 1.25 μM Rotenone (#R8875, Sigma-Aldrich) and 2-deoxyglucose (2-DG; #D8375, Sigma-Aldrich) were consecutively injected into the wells. The responses of the colonocytes to the different drug injections can then be used to calculate non-mitochondrial respiration, basal oxygen consumption rate (basal **OCR**), maximal respiration and spare respiratory capacity (**SRC**) from the oxygen consumption rate. Extracellular acidification rate (**ECAR**) is measured simultaneously. It can be corrected for the contribution of mitochondrial CO_2-production using the buffer capacity factor of the medium to approximate lactate flux, which is expressed as the glycolytic proton efflux rate (**glycoPER**).

Normalisation

Seahorse XF assays were normalised using an adaptation of a previously in-house generated R-script that uses the EBImage package available for Bioconductor (Pau et al., 2010; Janssen et al., 2021). The normalisation method was validated and optimised together with the enterocyte isolation protocol (Bekebrede et al., 2021). Brightfield images were obtained before the Seahorse XF assay, using the Cytation 1 (BioTek Instruments, Inc., Winooski, VT, USA), with a 4× objective. The images were processed as follows: a Gaussian blur low-pass filter was applied to generate a background image, followed by subtracting the background image from the original. The background-corrected image was then inverted to generate a "white-objects-on-black-background image". This image was subsequently cropped by 5% to remove potential noise from the XF assay plate moulded stops that are present on the plates to prevent the sensors from disrupting the cell monolayer. Images were then analysed to calculate pixel intensity values for all the pixels in the image. All the pixels with an intensity >1 were counted as representing the presence of a cell, and we refer to these as "cell pixels". For conversion of cell pixels back to cell numbers, an external calibration curve was generated, using a second-order polynomial fit analysis on data obtained from three individual pig colonocyte standard curves. These cell numbers were subsequently used for normalisation of the Seahorse XF assays.

Citrate synthase activity

To investigate mitochondrial density in colonic mucosa, we used the citrate synthase activity assay kit according to the manufacturer's instructions (#CS0720, Sigma-Aldrich). In short, intestinal scrapings were first crushed with a pestle in liquid nitrogen. The crushed scrapings were then added to lysis buffer containing 50 mM Tris-HCl pH 7.4 150 mM NaCl, 1% Triton X-100, 1 mM Ethylenediaminetetraacetic acid and protease and deacetylase inhibitors (2 μM trichostatin A, 10 mM nicotinamide and one tablet per 10 mL of both protease and phosphatase inhibitor cocktail (Roche)). Samples were first placed in a thermoshaker at maximum speed for 5 minutes in a cold room and were then submitted to a freeze–thaw cycle (−80 °C freezing, 4 °C thawing), after which they were sonicated on ice for 10 pulses of 2 seconds at 40 kHz. The sonicated samples were centrifuged at 10 000*g* and 4 °C to remove cell debris. The protein content was determined using the DC protein assay (Biorad), and protein content was equalised across all samples using 1x citrate synthase assay buffer. A baseline measurement was first taken, containing all relevant reagents and protein, but without oxaloacetate. Then, oxaloacetate was added and changes in absorbance at 412 nm were monitored for 30 minutes with a measurement interval of 24 seconds, using a Synergy HT Multi-detection microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The citrate synthase activity was then calculated using the change in absorbance in the linear section of the reaction according to the manufacturer's protocol, accounting for the sample volume, the pathway length and the protein input. Citrate synthase activity is expressed as units (μmol/ml/min) per mg protein.

Digesta sample analysis

Intestinal digesta was collected from the distal colon by clamping off the colon between 66 and 80%, and collecting the digesta within the intestinal segment. DM content of feed and freeze-dried matter content of digesta were determined according to the International Organisation for Standardisation (ISO) 6496 (International Organization for Standardization, 1999). Nitrogen in digesta was determined by using the Kjeldahl method according to ISO 5893 (International Organization for Standardization, 2005). TiO_2 was analysed in freeze-dried digesta samples according to (Myers et al., 2004). To determine SCFA concentrations in the samples, distal colon digesta samples were homogenised, and subsamples were acidified using an equal volume of Ortho-phosphoric acid (0.1 M, #100573, Merck), and were then stored at −20 °C until use. For the analysis, samples were thawed and centrifuged for 10 min at 4816*g* to obtain the liquid fraction. To this liquid fraction, 15.89 mM 2-methyl valeric acid was added as internal standard, and the mixture was centrifuged for 5 min at 20871*g* to remove any remaining debris. The clear supernatant (0.5 μL) was injected onto a gas chromatograph (Trace 1300, Thermo Fisher Scientific, Waltham, MA, USA) with a split/splitless injector operated in split mode (split ratio 1:36) and a temperature of 260 °C, using the HP-FFAP column (30 m × 0.32 mm × 0.25 μm, #19091F-413, Agilent J&W, Santa Clara, CA, USA) and hydrogen as mobile phase for detection by FID. Using this method, we analysed specific metabolites of primarily protein fermentation (isobutyrate, isovalerate, valerate and isocaproate) and primarily fibre fermentation (acetate, propionate, butyrate and caproate). Because we aimed to investigate how diets, through luminal metabolites in the distal colon, affected IEC metabolic function, we expressed metabolite concentration as a colonic flux in mmol per hour (see data analysis for calculations). Metabolite concentrations in mmol per kg digesta can be found in Supplementary Fig. S1.

Data analysis and statistics

For pig performance parameters, data are presented as mean with pooled SE. Data of metabolite analysis, metabolic function and isolation parameters are presented using bar graphs with mean ± SEM. For SCFA and N analysis, respectively, two and three samples were missing because they were exhausted. Two pigs were excluded from the viability and metabolic flux analysis because of an inability to normalise the data accurately. An additional eight pigs were absent from the analysis of cell yield because the volume of isolating medium was not recorded. The number of pigs included in each group is described in the figure and table legends. For pig performance parameters, data for pens were analysed. For metabolite fluxes and IEC isolations, data from individual pigs were analysed, and a random effect of pen was included to account for higher similarity within pens than between pens.

Mean retention time in hours was calculated using the following formula (de Vries and Gerrits, 2018):

$$\text{Mean retention time (h)} = 24 * \left(\frac{[T]_{sample} * [Q]_{sample}}{[T]_{diet} * \frac{Intake}{24h}} \right)$$

where $[T]_{diet}$ and $[T]_{sample}$ (g/kg) are TiO_2 in the diet and distal colon digesta, Q_{sample} (g) is the tracer pool size in the distal colon, and intake is feed intake per 24 h (kg).

Metabolite flow in mmol/hour was calculated using the following formula:

$$\text{metabolite flow} = [M]_{sample} * [Q]_{sample} / \text{mean retention time (h)}$$

where $[M]_{sample}$ (mmol/kg digesta) is metabolite concentration in distal colon digesta, and Q_{sample} (g) is the distal colon digesta pool size, obtained between 66 and 80% of the colon.

Net disappearance of nitrogen was calculated using the following formula:

$$\text{net N disappearance colon (g/day)} = [N]_{intake} * (\text{apparent total tract digestibility N} - \text{apparent ileal digestibility N})$$

where $[N]_{intake}$ is daily nitrogen intake per day (g), and apparent total tract digestibility and apparent ileal digestibility of nitrogen are in percent (%).

Normality of model residuals was checked using the Shapiro-Wilk test, and if assumptions were not met, data were transformed as indicated in the table and figure legends when applicable. Although model residuals were not normally distributed for isobutyrate and isovalerate due to an outlier, removing this outlier did not change the conclusions and was therefore not removed for the final analysis. If all assumptions were met, two-way ANOVA was performed with an α of 0.05, using protein source and fibre type as factors and pen as a random effect. In case of significant interaction between factors, a posthoc Tukey test was performed to determine differences between groups. To correlate metabolites to IEC metabolic flux, fermentation metabolites were subclassified as primary protein fermentation metabolites (ammonia, isobutyrate, isovalerate, valerate and isocaproate) and primarily fibre fermentation metabolites (acetate, propionate, butyrate and caproate). To ensure that metabolites with high concentrations did not disproportionately influence the overall values of protein- or fibre fermentation metabolites, all metabolites were rank-scaled before they were added up. Then, the protein- and fibre- fermentation metabolite fluxes were correlated to IEC metabolic flux parameters using Spearman correlation. All statistical analysis was performed using R version 4.1, and data were visualised using GraphPad v.9 (GraphPad Software, CA, USA).

Results

Effects of the feeds on pig performance

Nor slaughter weight, nor small and large intestinal length was affected by the dietary treatments (Table 2). Collagen-fed pigs had a reduced weight gain compared to whey-fed pigs (Table 2, $P < 0.01$).

Metabolite analysis in distal colon digesta

Collagen-fed pigs had reduced freeze-dried matter content of the distal colon digesta compared to whey-fed pigs, while the inclusion of high fermentable fibre increased distal colon freeze-dried matter content (Supplementary Fig. S1a). We found that the net colonic nitrogen disappearance was increased by 135% in the collagen-protein compared to the whey-protein diets (Fig. 1a). The dietary treatments did not affect the mean retention time in the distal colon (Fig. 1b). The collagen-fed pigs had an increased colonic flux of the protein fermentation metabolites ammonia, isobutyrate, isovalerate and valerate compared to the whey-fed pigs (Fig. 1c–g) as well as an increased acetate flux (Fig. 1h). Feeding high fermentable fibre increased colonic fluxes of all analysed SCFA's, both fibre fermentation-derived metabolites (acetate, propionate, butyrate and caproate, Fig. 1h–k), and those related to protein fermentation (ammonia, isobutyrate, isovalerate, valerate and isocaproate, Fig. 1c–g).

Intestinal cell isolation parameters

Regardless of the fibre treatment, feeding pigs with collagen-protein, compared to whey-protein, increased the viability of isolated colonocytes by 12% (Fig. 2a, protein $P < 0.01$). In contrast, the yield of cells/cm^2 was reduced by 32% in isolations of pigs fed the high-fibre diets, regardless of the protein source (Fig. 2b, fibre $P < 0.05$).

Table 2
Effect of two weeks of feeding diets differing in protein source and fibre level on performance and intestinal traits in pigs. Data are presented as mean with pooled SE, n = 2 pens per group.

	Whey		Collagen					
	Low Fibre	High Fibre	Low Fibre	High Fibre	SE	P_{fibre}	$P_{protein}$	$P_{interaction}$
Start weight (kg)	23.4	22.4	22.6	22.8	0.7	0.46	0.71	0.27
Daily weight gain (g/day)	355	365	286	258	36	0.99	<0.05	0.31
Slaughter Weight (kg)[1]	29	28.1	27.1	26.8	0.7	0.23	0.06	0.28
Length Small Intestine (m)	14.6	14	14.8	13.9	0.5	0.10	0.81	0.70
Length Large Intestine (cm)	265	274	282	248	19	0.38	0.73	0.18

[1] Slaughter weight data were transformed using exponential transformation approach normal distribution; reported values are back-transformed.

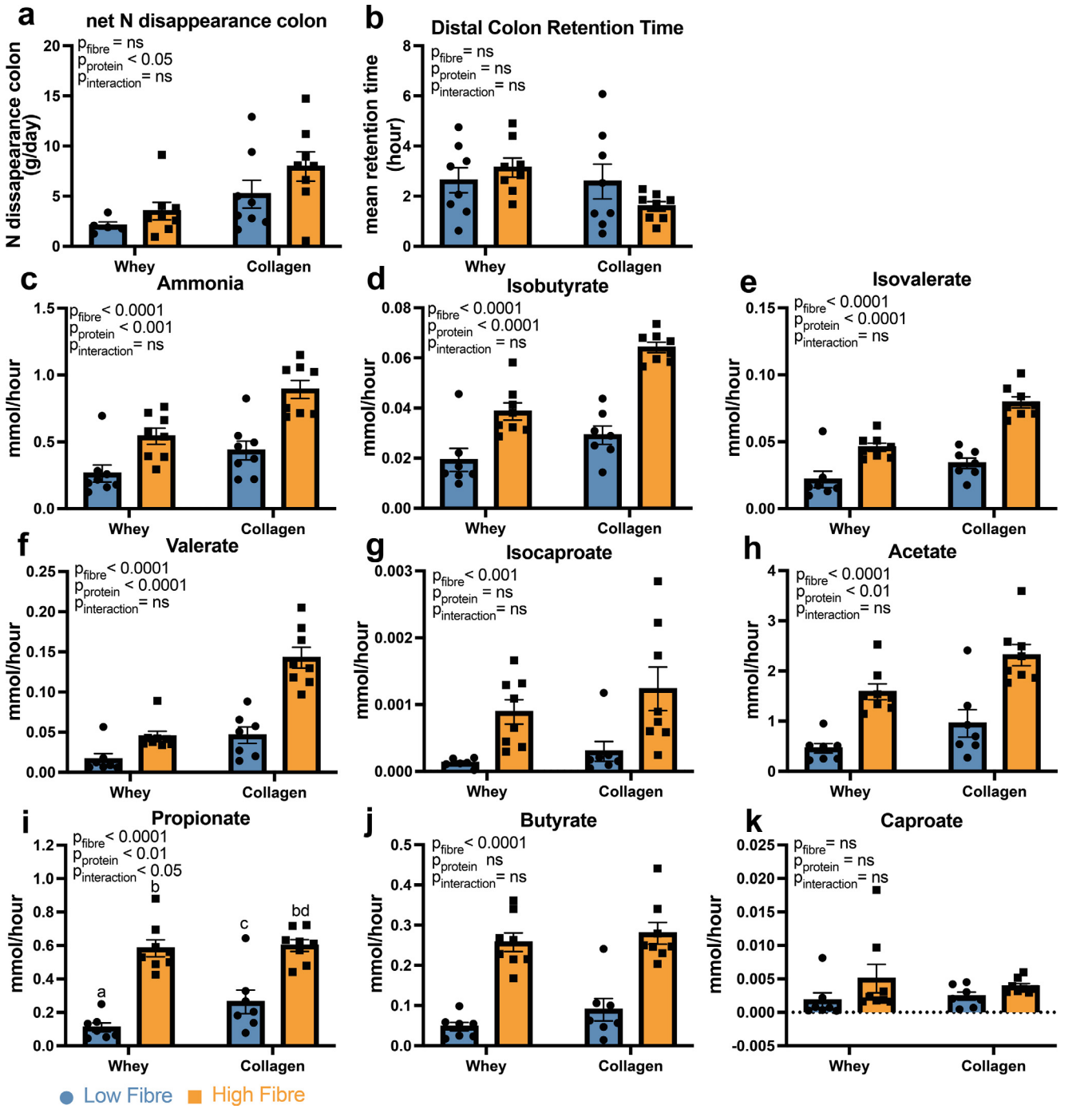

Fig. 1. Distal colon digesta metabolite levels after two weeks of feeding pigs diets varying in dietary protein source and fibre level. Net nitrogen disappearance in the colon (a). Mean retention time in distal colon (b). Protein fermentation metabolites ammonia (c), isobutyrate (d), isovalerate (e), valerate (f) and isocaproate (g) and fibre fermentation metabolites acetate (h), propionate (i), butyrate (j) and caproate (k) were analysed in distal colon digesta of pigs following two weeks on the four feeds. The levels were corrected for mean retention time, yielding intestinal fluxes in mmol per hour. Bars represent mean ± SEM. For panel a, n = 5 for low-fibre whey group, n = 8 for all other groups. For panel b, n = 8 for all groups, for panels c–k, n = 7 for low-fibre whey and collagen groups, n = 8 for high-fibre whey and collagen groups. Significance was analysed using two-way ANOVA, and results are presented in the top left corner of each graph. Similar letters indicate no significant differences between groups in case of significant interaction effects, as analysed using Tukey's posthoc analysis. Data for nitrogen disappearance, butyrate, acetate, propionate, valerate and caproate were log-transformed to approach normal distribution.

Extracellular metabolic flux analysis of isolated pig colonocytes

The dietary interventions did not affect the basal mitochondrial respiration of IECs (Fig. 3a). Colonocytes of high fermentable fibre-fed pigs in the whey-protein diet, but not in the collagen-protein diet had a reduced mitochondrial capacity, as can be seen by the significant reduction of maximal respiration by 35% (Fig. 3b, interaction $P < 0.05$) and the significant reduction in spare respiratory capacity by 20% (SRC, Fig. 3c, interaction $P < 0.05$). IECs (i.c. colonocytes) from high fermentable fibre-fed pigs had a 37% decreased glycoPER compared to IECs of low fermentable fibre-fed pigs (Fig. 3d, $P < 0.001$). The energetic phenotype plots (Fig. 3e–f) show an overall decrease in metabolic function of IECs in high fermentable fibre upon inclusion of fermentable fibre in a

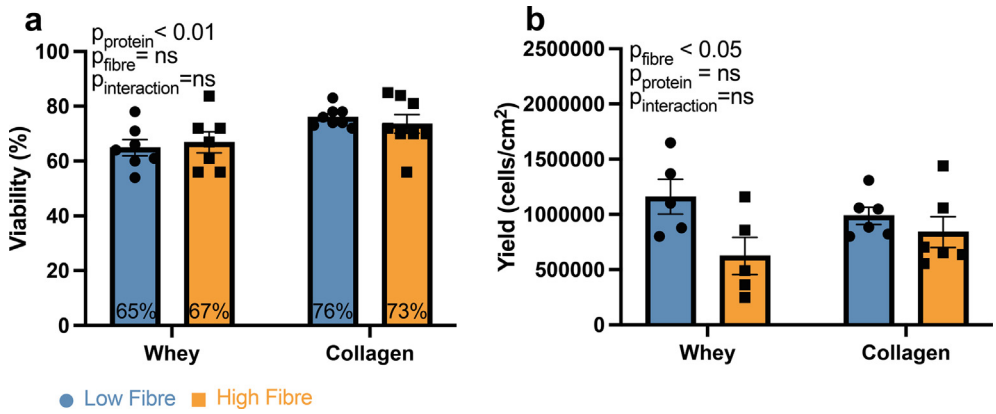

Fig. 2. Intestinal epithelial cell isolation parameters after two weeks of feeding pigs diets varying in dietary protein source and fibre level. Viability as percentage of dead cells over total cells (a; n = 7 for whey-protein groups and n = 8 for collagen-protein groups) and cell yield expressed as viable cells isolated per cm^2 intestinal tissue (b; n = 5 for whey-protein groups and n = 6 for collagen-protein groups) were calculated following isolation from the colonic segments. Bars represent mean ± SEM. Significance was analysed using two-way ANOVA, and results are presented in the top left corner of each graph. Similar letters indicate no significant differences between groups in case of significant interaction effects, as analysed using Tukey's posthoc analysis.

whey-protein-based feed (Fig. 3e), while high levels of fermentable fibre in a collagen-protein-based feed mainly reduced glycolytic function of isolated pig colonocytes (Fig. 3f). Mucosal citrate synthase activity in colonocyte scrapings was determined as an indicator of mitochondrial density, and this was not affected by the dietary treatments (Fig. 4).

Discussion

Intestinal epithelial cells rely on cellular energy production through glycolytic and oxidative pathways to maintain their various functions (Yang et al., 2016; Zhou et al., 2018). Factors that affect epithelial energy production, such as metabolites produced from feedstuffs by the microbiome, may thus have far-reaching consequences for intestinal homeostasis. In this paper, we showed functionally that diet indeed affected the metabolism of isolated pig colonocytes, and observed an interaction between protein source and fibre level. We demonstrated that the mitochondrial capacity of isolated IECs was reduced by fermentable fibre only in whey-protein diets. At the same time, the glycolytic function of IECs was reduced by high fermentable fibre, irrespective of dietary protein source. Especially in the well-digestible whey-protein diets, we thus observed an overall reduction of metabolic capacity upon the addition of high fermentable fibre to the diet. These findings gave us valuable insights into the interaction between various dietary components.

Possible implications of altered colonocyte metabolic function

In our study, pigs were growing at expected rates considering the nutritional strategies imposed: a reduction of digestible protein in the collagen diets was reflected in a lower rate of bodyweight gain. Apart from this difference in daily weight gain, no clear differences in pig performance parameters were observed. However, we show that the diets did affect colonocyte capacity function. One of the hallmarks of good intestinal health is a well-functioning intestinal barrier. To maintain this barrier, sufficient ATP needs to be produced to allow for intestinal tight junction assembly and maintenance of intestinal barrier integrity (Bjarnason and Takeuchi, 2009; JanssenDuijghuijsen et al., 2017), and the decreased metabolic capacity that was observed in pigs fed a high-fibre diet could therefore impact barrier function. Furthermore, it has been shown that elevated oxygenation of the colonic lumen due to lower oxygen consumption in the colonocytes could allow for higher pathogenic bacterial growth (Byndloss et al., 2017; Cevallos et al., 2019). In our study, we identified lower mitochondrial oxygen consumption capacity, which could translate into higher lumen oxygen levels and possibly higher pathogenic bacterial growth.

Effect of dietary interventions on colonocyte metabolic function and microbial metabolite production

Although dietary fibre is often considered beneficial, partly by providing substrates that increase mitochondrial function and intestinal energy production, some studies indicate a reducing effect of high concentrations of SCFAs on intestinal barrier function and IEC metabolism. For example, *in vivo* studies in rats showed that feeding fructo-oligosaccharides decreased intestinal barrier function (Ten Bruggencate et al., 2005; Rodenburg et al., 2008; Genda et al., 2017). In addition, especially *in vitro* studies have demonstrated that high concentrations of e.g. butyrate can induce apoptosis (Peng et al., 2007; Matthews et al., 2012). These findings challenge the view that dietary fibres are always beneficial for intestinal health and function. In our study, the decrease in metabolic capacity induced by high-fibre feeds was especially prominent in the whey-protein feeds. An explanation for this could be that because of the high digestibility of whey-protein, less protein enters the colon, which is thus less available as a colonic energy substrate (Fig. 1a). To increase the efficient use of the remaining substrates, mitochondrial capacity should be induced to most optimally obtain energy from the limited remaining substrates (Rodenburg et al., 2008). This is consistent with our observation of higher metabolic capacity of IECs from pigs fed with low-fibre whey-protein diets (Fig. 3). Furthermore, we observed a lower spare respiratory capacity of IECs isolated from pigs fed the collagen-protein diets, possibly obscuring the negative impact of high fibre in these diets. How collagen diets decreased spare respiratory capacity could be explained by the increased nitrogen flow into the colon, in addition to increased fluxes of microbial metabolites that could potentially harm mitochondrial function. Protein fermentation metabolites, such as H_2S, ammonia and others, have indeed been shown to impact mitochondria (Darcy-Vrillon et al., 1996; Blachier et al., 2007; Libiad et al., 2019).

Interestingly, we did not find an interaction effect of protein source and fibre level for glycoPER and instead found that high fibre decreased colonocyte glycoPER irrespective of protein source. High-fibre diets were reported to increase intestinal proliferation

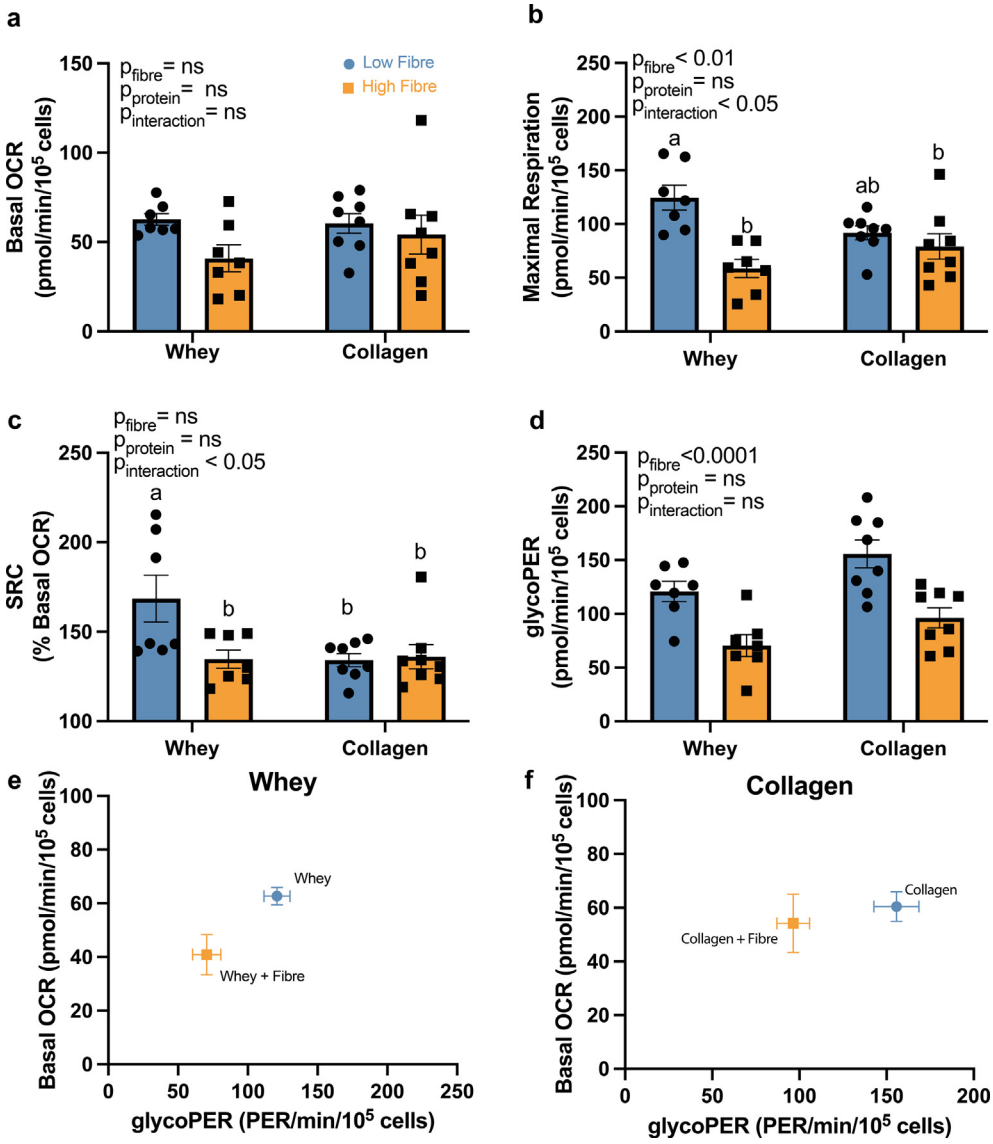

Fig. 3. Extracellular metabolic flux analysis of isolated pig colonocytes after two weeks of feeding pigs diets varying in dietary protein source and fibre level. Mitochondrial oxygen consumption is represented by basal oxygen consumption (a), maximal respiration (b) and spare respiratory capacity (c). Glycolytic function is measured using glycoPER (d). Energetic profile plots for the whey-protein based (e) and collagen-protein based (f) feeds show the changes in basal metabolic function upon the inclusion of fermentable fibre. *N* = 7 for the whey-protein diets and n = 8 for the collagen-protein diets. Bars represent mean ± SEM. Significance was analysed using two-way ANOVA, and results are presented in the top left corner of each graph. Similar letters indicate no significant differences between groups in case of significant interaction effects, as analysed using Tukey's posthoc analysis. Abbreviations: OCR = oxygen consumption rate, SRC = spare respiratory capacity, glycoPER = glycolytic proton efflux rate.

(Jin et al., 1994), crypt depth (Mentschel and Claus, 2003; Serena et al., 2007; Zaworska-Zakrzewska et al., 2020) and mucus layer thickness (Barcelo et al., 2000; Willemsen et al., 2003), potentially resulting in increased percentages of differentiated colonocytes in the isolated cell population. Differentiated colonocytes are thought to be less glycolytic, while the transit-amplifying cells, which are their precursors, rely more on glycolysis (Fan et al., 2015; Yang et al., 2016; Litvak et al., 2018). Thus, a larger proportion of differentiation colonocytes in the isolated IEC population could underlie the decreased glycoPER in high-fibre-fed pigs. Although the lowered glycolytic function of differentiated colonocytes was shown to be due to elevated mitochondrial function, we did not observe elevated mitochondrial function, possibly because the mitochondrial oxidative capacity may be driven by fatty acids, or even SCFAs like butyrate, which we did not add extracellularly during our metabolic flux analysis. Since the glycolytic function is not dependent on SCFAs, we observed the effects of high-fibre feeding irrespective of protein source.

In this study, our primary aim was to investigate whether colonocyte metabolism was altered in response to dietary interventions. An important route for interaction between diet and colonocytes is through microbial metabolites. Since the speed with which metabolites, contained in digesta, travel through the colon could affect the exposure of colonocytes to these metabolites, we calculated colonic fluxes of the metabolites. We expected that the poorly digestible protein would increase the flux of protein-derived metabolites, while including high levels of fermentable fibre would decrease the flux of protein-derived metabolites and increase the fluxes of acetate, butyrate, propionate and caproate. Indeed, we found that high-fibre feeds significantly increased the concentration of these fibre fermentation metabolites. However, the high-fibre diets also increased the flow of the protein fermentation metabolites ammonia, isobutyrate, isovalerate, valerate and isocaproate. Even though protein fermentation in high-fibre diets is not always reported, valerate has been shown to increase upon high-fibre feeding in pigs and other species (Zhao et al., 2018;

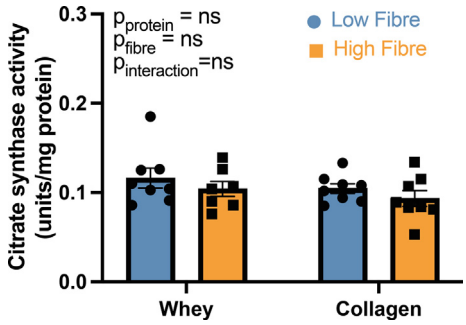

Fig. 4. Citrate synthase activity in distal colon mucosal scrapings after two weeks of feeding pigs diets varying in dietary protein source and fibre level. Bars represent mean ± SEM, n = 7 for low-fibre whey, n = 6 for high-fibre whey and n = 8 for both collagen groups. Significance was calculated using two-way ANOVA on log-transformed data.

Tuśnio et al., 2020; Zaworska-Zakrzewska et al., 2020; Hao et al., 2021). This is possibly due to an increased microbial richness and stability (Tap et al., 2015), which could benefit not only fibre-fermenting communities but also the proteolytic ones (Xu et al., 2021). Additionally, high-fibre feeds could have led to increased mucus production, which consists of protein for about 20%, and could thus also increase the flow of protein fermentation products (Barcelo et al., 2000; Willemsen et al., 2003; Raimondi et al., 2021).

To investigate whether the colonic metabolite fluxes correlated with IEC metabolic function, we pooled the metabolite fluxes into two groups: primarily protein fermentation-derived metabolites (ammonia, isobutyrate, isovalerate, valerate and isocaproate), and primarily fibre fermentation-derived metabolites (acetate, propionate, butyrate and caproate). We found comparable negative correlations between the protein- or fibre-derived fermentation metabolites and mitochondrial capacity parameters maximal respiration and spare respiratory capacity (r = −0.46 and −0.63; r = −0.40 and −0.40, respectively, Supplementary Table S1). In addition, fibre-derived metabolites correlated negatively with the glycolytic capacity of colonocytes (r = −0.55, Supplementary Table S1). Thus, we found that metabolites present in the intestinal lumen negatively correlate to metabolic function of pig colonocytes, suggesting that microbial metabolites could have played a role in the decreased metabolic function of IECs, and potentially affected intestinal health through this route.

Methodological improvements and study limitations

We have taken several steps to ensure accurate measurement of IEC metabolic function. Firstly, citrate synthase was analysed as a marker for mitochondrial density. The observed unaltered citrate synthase levels ensure that the observed changes in energy metabolism were not due to an altered mitochondrial density, but were true alterations in the metabolic function of the IECs (Fig. 4, (Vigelsø et al., 2014)). Secondly, IEC viability was measured to investigate whether this was affected by the diets, since a decreased viability has been associated with a reduced mitochondrial metabolism (Osellame et al., 2012). We did observe lower viability in both groups fed a whey-protein diet, but only in the fibre-containing group, metabolism was decreased, rendering it unlikely that the metabolic alterations were due to decreased IEC viability. Isolated IEC yields were lower in the high-fibre diets. This may be due to the effects of the fibre fermentation metabolites on intestinal proliferation (Jin et al., 1994), crypt depth (Mentschel and Claus, 2003; Serena et al., 2007; Zaworska-Zakrzewska et al., 2020) and thickness of the mucosal layer (Barcelo et al., 2000; Willemsen et al., 2003). It is however unlikely that differences in cell yield could have contributed to the functional metabolic differences that were observed, since an equal number of cells were plated for metabolic analysis, and on top of this, the cell numbers in the assay wells were quantified, and the data were normalised for this. In conclusion, neither difference in viability, yield or mitochondrial density can explain the altered metabolic function of colonocytes in response to the diets.

A limitation of this study is that the metabolism of isolated colonocytes is evaluated only in the presence of glutamine, glucose and pyruvate. The use of multiple substrates is important since substrate preference could be altered by the diets, as was shown in several studies in both pigs and rats (Darcy-Vrillon et al., 1993; Marsman and McBurney, 1995; Fleming and Gill, 1997). In our study, it cannot be excluded that IECs from the high fermentable fibre-fed pigs have an increased dependency on one of the SCFAs, which were not included as substrates in the metabolic analysis. A second limitation is that we did not assess possible alteration in IEC composition. Altered composition of the cell population could impact the results, because each intestinal cell type displays a distinct metabolic phenotype (Ho and Theiss, 2022), and it would thus be relevant and interesting to specifically analyse possible alterations in the composition of the isolated IEC population to better interpret the results.

Conclusions

In conclusion, we found that diets affect the metabolism of isolated pig colonocytes, and there was an interaction between protein source and fibre level. Glycolytic activity was reduced in colonocytes isolated from high-fibre-fed pigs, irrespective of protein source, while metabolic capacity was reduced only in high-fibre-fed pigs in a whey-protein diet. Metabolic analysis of isolated pig colonocytes could be a sensitive tool to further assess the functional metabolic effects of dietary interventions. This is important because intestinal epithelial energy metabolism is tightly linked to cell function and may impact intestinal health and function.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2022.100625.

Ethics approval

All experimental procedures were approved by the Dutch Central Committee of Animal Experiments (the Netherlands) under the authorisation number AVD1040020171667.

Data and model availability statement

All data generated or analysed during this study are available from the corresponding author upon reasonable request. None of the data was deposited in an official repository, but the R-script for brightfield analysis of microscopy image and data normalisation of Seahorse XF Extracellular Flux analysis is available from GitHub (https://github.com/vcjdeboer/seahorse-data-analysis-PIXI).

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Declaration of interest

The authors declare no conflict of interest.

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