Research Paper

Characterization of butyrate transport across the luminal membranes of equine large intestine

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New Findings

- **What is the central question of this study?**
  Butyrate, a product of colonic microbial fermentation of dietary fibre (grass), is a major source of energy for the horse and plays an important role in maintaining the health of the intestine. What are the properties of the membrane protein and what is the mechanism by which butyrate is absorbed in equine large intestine (colon)?
- **What is the main finding and its importance?**
  We have identified the mechanism of and membrane protein involved in butyrate transport in equine large intestine. This knowledge will allow rational approaches to the design of dietary formulations to enhance butyrate production and absorption in equine colon, in order to provide more energy for the horse and maintain its gut health.

The diet of the horse, pasture forage (grass), is fermented by the equine colonic microbiota to short-chain fatty acids, notably acetate, propionate and butyrate. Short-chain fatty acids provide a major source of energy for the horse and contribute to many vital physiological processes. We aimed to determine both the mechanism of butyrate uptake across the luminal membrane of equine colon and the nature of the protein involved. To this end, we isolated equine colonic luminal membrane vesicles. The abundance and activity of cysteine-sensitive alkaline phosphatase and villin, intestinal luminal membrane markers, were significantly enriched in membrane vesicles compared with the original homogenates. In contrast, the abundance of GLUT2 protein and the activity of Na^+–K^+-ATPase, known markers of the intestinal basolateral membrane, were hardly detectable. We demonstrated, by immunohistochemistry, that monocarboxylate transporter 1 (MCT1) protein is expressed on the luminal membrane of equine colonocytes. We showed that butyrate transport into luminal membrane vesicles is energized by a pH gradient (out < in) and is not Na^+ dependent. Moreover, butyrate uptake is time and concentration dependent, with a Michaelis–Menten constant of 5.6 ± 0.45 mM and maximal velocity of 614 ± 55 pmol s^{-1} (mg protein)$^{-1}$. Butyrate transport is significantly inhibited by *p*-chloromercuribenzoate, phloretin and α-cyano-4-hydroxycinnamic acid, all potent inhibitors of MCT1. Moreover, acetate and propionate, as well as the monocarboxylates pyruvate and lactate, also inhibit butyrate uptake. Data presented here support the conclusion

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that transport of butyrate across the equine colonic luminal membrane is predominantly accomplished by MCT1.

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Introduction

Horses are trickle feeders whose natural diet is pasture forage (grass). They possess a voluminous and elaborate large intestine containing a microbial population uniquely adapted to hydrolyse dietary plant fibre to soluble sugars, which are subsequently fermented to monocarboxylates, notably acetate, propionate and butyrate, referred to as short-chain fatty acids (SCFAs). A large proportion (60–70%) of the horse's body energy is provided by SCFAs absorbed from the caecum and colon (Argenzio & Hintz, 1972; Bergman, 1990). In normal circumstances, very little lactate is present; however, in horses fed high-grain diets or those suffering from some forms of dietary-induced intestinal disease, there are significantly higher concentrations of lactic acid in the colonic contents, disposing the horse to lactic acidosis and being detrimental to pH-sensitive microbiota (Daly *et al.* 2012).

Butyrate serves as the major respiratory fuel for colonic epithelial cells (Roediger, 1982), enhances mucosal barrier integrity by downregulating expression of pro-inflammatory genes (McDermott & Huffnagle, 2014) and is essential to the maintenance of homeostasis in the colonic epithelium (Bugaut & Bentéjac, 1993; Treem *et al.* 1994; Hamer *et al.* 2008). As such, butyrate is of fundamental importance to the health of colonic mucosa, and alteration in its luminal production or supply is associated with a variety of colonic disorders (Soergel, 1994; Thibault *et al.* 2007). Propionate is predominantly used by the liver as the precursor for gluconeogenesis, while acetate is used as an energy source by the peripheral tissues and is a precursor for lipogenesis (Cummings & Macfarlane, 1997).

A number of mechanisms for SCFA absorption across the apical (luminal) membrane of colonocytes in various species have been postulated. Diffusion of SCFAs in protonated form has been proposed as one route for SCFA absorption in rat and rabbit colon (Charney *et al.* 1998). However, at the colonic luminal pH of ∼7.5 (Medina *et al.* 2002; Julliand *et al.* 2001) SCFAs exist almost entirely in their dissociated forms, the cellular entry of which is dependent on a specific carrier protein. Furthermore, in the majority of studies, SCFA transport follows saturation kinetics and is inhibitable, characteristics consistent with a carrier-mediated process.

Investigations using membrane vesicles isolated from rat colon have suggested that SCFAs are absorbed by a $SCFA^-$–HCO_3^- exchanger (Mascolo *et al.* 1991). This process is neither Na^+ dependent nor coupled to a Cl^-–HCO_3^- exchanger (McNeil *et al.* 1979; Mascolo *et al.* 1991; Kawamata *et al.* 2007). However, to date, the transport protein responsible for $SCFA^-$–HCO_3^- exchange has not been identified.

Another proposed mechanism for SCFA transport across the colonic luminal membrane is via the Na^+-coupled monocarboxylate cotransporter 1 (SMCT1). Microinjection of SMCT1 cRNA into oocytes led to the increased ability of oocytes to transport SCFAs in a Na^+-dependent manner (Miyauchi *et al.* 2004) and, using immunohistochemistry, Gopal *et al.* (2007) demonstrated that SMCT1 protein is expressed on the apical membrane of mouse jejunum, ileum and colon.

More significantly, however, a number of independent studies have shown that transport of butyrate across the human, pig and chicken colonic luminal membrane (Tamai *et al.* 1995; Ritzhaupt *et al.* 1998*a*; Hadjiagapiou *et al.* 2000; Cuff *et al.* 2005; Gill *et al.* 2005; Borthakur *et al.* 2012) and also in the human intestinal cell lines, Caco-2 and AAC1, is accomplished via a H^+–butyrate symport mechanism (Stein *et al.* 2000; Hadjiagapiou *et al.* 2000; Cuff *et al.* 2002; Martín-Venegas *et al.* 2007; Lecona *et al.* 2008; Gonçalves *et al.* 2009) and suggested that this H^+–butyrate symporter is the monocarboxylate transporter 1 (MCT1). Western blot analysis has shown that MCT1 protein is expressed on the luminal membrane of colonic epithelial cells and that butyrate uptake is inhibited by specific inhibitors of MCT1 (Ritzhaupt *et al.* 1998*b*; Hadjiagapiou *et al.* 2000). Furthermore, microinjection of MCT1 cRNA into *Xenopus laevis* oocytes resulted in the expression of MCT1 protein in the plasma membrane of oocytes. The expressed protein was capable of transporting L-lactate as well as butyrate (Ritzhaupt *et al.* 1998*b*), supporting the role of MCT1 in intestinal butyrate transport. Moreover, expression of MCT1 has also been shown to be enhanced by increased luminal concentrations of butyrate (Cuff *et al.* 2002, 2005). Notably, Garcia and co-workers, who functionally expressed MCT1 cDNA in a human breast cancer cell line (MDA-MB-231; Garcia *et al.* 1994*a*) and in Chinese hamster ovary cells (Garcia *et al.* 1994*b*), showed that

lactate uptake into these cells was stimulated by prior loading of cells with anions. There are some controversies regarding the location of MCT1 either on the luminal and/or the basolateral membrane (Shimoyama *et al.* 2007; Kirat *et al.* 2009).

Considering the importance of SCFAs as the major energy source for the horse and the important role of butyrate in maintaining the gut health, we aimed to identify the mechanism and the nature of SCFA (butyrate) transport across the equine colonic luminal membrane. Using a technique for isolating luminal membrane vesicles, well established in our laboratory (Ritzhaupt *et al.* 1998*a*; Dyer *et al.* 2009), we prepared and characterized membrane vesicles from equine colonic tissues. We demonstrated that these membrane vesicles were enriched in cysteine-sensitive alkaline phosphatase and villin, classical markers of the colonic luminal membrane (Brasitus & Keresztes, 1984; West *et al.* 1988, Pinto *et al.* 1999), whereas activity of Na^+–K^+-ATPase and abundance of Glucose Transporter isoform 2 (GLUT2), markers of the basolateral membrane (Forbush, 1983; Pinches *et al.* 1993), were negligible in membrane vesicles compared with the original cellular homogenates. This pattern of expression confirms that the membrane vesicles originate from the colonic luminal membrane and are devoid of contamination by the basolateral membrane. We showed that butyrate transport into these equine colonic luminal membrane vesicles (LMVs) was enhanced in the presence of a pH gradient (extravesicular < intravesicular) and that intravesicular inclusion of bicarbonate enhances the rate of H^+-dependent butyrate transport. By immunohistochemistry, we demonstrated that MCT1 protein is expressed on the luminal membrane of equine colonocytes with a similar profile to that of villin.

Understanding the mechanism involved in the uptake of butyrate across the equine large intestine will allow rational formulation of feed to enhance butyrate production, with subsequent effects on increased MCT1 expression and SCFA absorption.

Methods

Ethical approval

Human intestinal biopsies were obtained with ethical permission and patients' consent (Liverpool Research Ethical Committee REF: 02/11/228/A & 2K/128).

Tissue collection and storage

Horse colonic tissue samples were obtained from six freshly slaughtered horses from the local abattoir, euthanized by captive bolt. The horses were adult (7- to 12-year-old) mares and geldings of various breeds. They had been maintained on pasture forage in the mid-Cheshire area (UK) with *ad libitum* access to mature grass comprising mainly *Lolium perenne* [average nutritional value 8.5 kJ of digestible energy (g dry matter)$^{-1}$]. Sections of ventral colon were rapidly removed and washed with ice-cold saline (0.9% w/v NaCl, pH 7.0) to discard faecal contents. The tissue was gently blotted with paper towel to remove mucus before the surface epithelial cells were removed by scraping the mucosa with a glass slide. The scrapings were wrapped in aluminium foil and immediately frozen in liquid nitrogen before storage at −80°C.

Preparation of equine colonic luminal membrane vesicles

Colonic luminal membrane vesicles were isolated as described by Ritzhaupt *et al.* (1998*a*) and Dyer *et al.* (2009). All steps were performed at 4°C. The mucosal scrapings from equine colon were defrosted in a solution containing 100 mM mannitol, 2 mM Hepes–Tris (pH 7.1), 0.2 mM benzamidine and 0.2 mM phenolmethylsulfonyl fluoride (PMSF)at 1 g scraping per 10 ml buffer. They were then homogenized for 1 min using a polytron (Ystral, Scientific Instruments, Cambridge, UK). The homogenate was filtered through nylon gauze to eliminate excess mucus. A sample of filtered homogenate (referred to as original homogenate) was retained for further analysis. Magnesium chloride (10 mM final concentration) was added to the remaining solution with continuous gentle stirring on ice for 20 min, before centrifugation at 1000*g* for 15 min (Sorvall RC5C SS-34 rotor; Fisher Scientific UK Ltd, Loughborough, UK). The resulting pellet was discarded and the supernatant filtered through gauze a second time, to remove any remaining fat, before centrifugation at 30,000*g* for 25 min. The resulting supernatant was discarded and the pellet collected and resuspended in a buffer containing 100 mM mannitol, 20 mM Hepes–Tris (pH 7.4) and 0.1 mM $MgSO_4$. The pellet was then homogenized using 40 strokes in a Dounce homogenizer (Jencons, East Ginstead, UK) with a tight-fitting Teflon pestle. This homogenate was centrifuged at 30,000*g* for 35 min and the final pellet resuspended in 500 μl of buffer containing 300 mM mannitol, 20 mM Hepes–Tris (pH 7.5) and 0.1 mM $MgSO_4$ and homogenized by passing through a 27-gauge needle several times. The resulting membrane vesicle suspension was aliquoted and stored in liquid nitrogen until use.

Preparation of postnuclear membrane fractions from human colonic biopsies

Postnuclear membranes were prepared based on the procedure described by Lambert *et al.* (2002). Normal human colonic biopsies, 30–60 mg wet weight, were homogenized using a polytron probe (6T microshaft;

Ystral) with 500 μl of a buffer (100 mM mannitol and 2 mM Hepes–Tris, pH 7.1). The probe was washed with a further 250 μl of the same buffer, added to the suspension, and centrifuged at 500*g* for 10 min (Sorvall RC5C). The supernatant was centrifuged at 30,000*g* for 30 min and the resulting pellet resuspended in a buffer containing 300 mM mannitol, 20 mM Hepes–Tris, pH 7.4, and 0.02% NaN_3, and made homogeneous with a Hamilton syringe. Aliquots of membranes were stored in liquid nitrogen until use.

Estimation of protein

Protein concentration was estimated by its capacity to bind Coomassie Blue, according to the manufacturer's instructions (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Porcine γ-globulin (30–150 μg protein) was used as the standard.

Marker enzyme assay

Cysteine-sensitive alkaline phosphatase. The activity of cysteine-sensitive alkaline phosphatase, a luminal membrane marker, was measured according to the procedure of Brasitus & Keresztes (1984). Equine colonic cellular homogenates and LMVs, 50 μg protein each, were incubated at 38°C for 5 min. The reaction was started by the addition of 900 μl of incubation buffer [32 mM glycine (pH 9.3), 3.2 mM $MgCl_2$, 0.32 mM $ZnSO_4$, 15 mM para-phenolnitrophosphate, ±10 mM L-cysteine]. The reaction was stopped after 15 min by the addition of 2 ml 1 M NaOH, and absorption (millimolar extinction coefficient = 17 mM^{-1} cm^{-1}) was read at 410 nm. The activity of cysteine-sensitive alkaline phosphatase was determined as the difference between specific activity in the presence and absence of L-cysteine.

Na^+–K^+-ATPase. The activity of Na^+–K^+-ATPase, a classical basolateral membrane marker, was measured according to the procedure of Forbush (1983). Saponin (0.05% w/v final concentration) was added to 50 μg protein per assay of either equine colonic cellular homogenate or LMVs and incubated at 38°C. Given that the natural tendency of any membrane is to seal itself upon disruption, saponin is used to permeabilize any sealed membranes, allowing access of the substrate. The reaction was started by the addition of either 400 μl of solution A [120 mM NaCl, 60 mM Tris–HCl (pH 7.5), 20 mM KCl, 4 mM Na_2ATP and 2 mM EDTA], for total ATPase activity, or solution B (solution A containing 5 mg ml^{-1} ouabain), for ouabain-sensitive ATPase activity. The reaction was stopped after 10 min by the addition of 1 ml of developing solution (1.15 M H_2SO_4, 1% w/v ammonium molybdate and 4% w/v ferrous sulfate) and left to develop for 1 h. A standard curve of K_2HPO_4 (0–720 μM) was prepared to calculate inorganic phosphate (P_i) release and developed at the same time as the samples. Absorption of standards and samples was read at 690 nm normalized against buffer blanks of solutions A or B, with specific activity being expressed as P_i released in nanomoles per minute per milligram of protein. The specific activity of Na^+–K^+-ATPase was determined by subtracting the specific activity determined using solution B from that measured using solution A.

Western blotting

Western blotting was carried out as previously described by Cuff *et al.* (2002). Protein contents of membrane vesicles were separated on 8% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS before being electro-transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The polyvinylidene difluoride membranes were blocked, for 1 h, with a solution (PBS containing 0.05% v/v Tween-20 and 5% w/v non-fat dried milk) before incubating, for 1 h, with primary antibodies to MCT1 (1:2000), GLUT2 (1:500) and SMCT1 (1:500), diluted in PBS-TM (PBS containing 0.05% v/v Tween-20 and 1% w/v non-fat dried milk).

The antibody to MCT1 was raised in rabbits (custom synthesis) to a synthetic peptide (CQKDTEGGPKEEESPV) corresponding to amino acids 485–500 of the human MCT1 C-terminus region as described previously (Cuff *et al.* 2002). We have shown in a number of our previous studies that this antibody reacts with a specific protein with apparent molecular mass of 40 kDa (Cuff *et al.* 2002, 2005; Lambert *et al.* 2002). We have cloned and sequenced equine MCT1 (accession number AY457175) and deduced the amino acid sequence (accession number AAR21622). The peptide sequence to which the human MCT1 antibody was raised shares 69% homology with equine MCT1. We have also cloned and sequenced cDNA encoding for equine GLUT2 (accession number AJ715983) and have deduced the amino acid sequence (accession number CAG29734; Dyer *et al.* 2009). The antibody to equine GLUT2 was raised in rabbits (custom synthesis) to a synthetic peptide (AAVEMEFLGATETA) corresponding to amino acids 523–536 of the equine GLUT2 C-terminus region. The polyclonal antibody to human SMCT1, raised in rabbits, was purchased from Novus Biologicals Ltd (Cambridge, UK). The peptide sequence to which the human SMCT1 antibody was raised shares 78% homology with the corresponding horse sequence. Postnuclear membranes isolated from normal human colonic biopsies, obtained during the routine course of gastrointestinal investigation, were used as a positive control for SMCT1 expression; human colon expresses SMCT1 (Gopal *et al.* 2007).

After washing (three times, each for 10 min), the membranes were incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Dako AC, Cambridge, UK). Immunoblots were developed using the WEST-one Western blot detection system (iNtRON Biotechnology, Chembio Ltd, St Albans, UK) and Bio-Max Light Chemiluminescence Film (Sigma-Aldrich, Poole, UK). The intensity of the immunoreactive bands was quantified using scanning densitometry (Phoretix 1D quantifier; Non-linear Dynamics, Newcastle-upon-Tyne, UK).

For assessment of villin expression, polyvinylidene difluoride membranes were stripped by three 10 min washes in 137 mM NaCl, 20 mM glycine hydrochloride (pH 2.5) and then re-probed with a monoclonal antibody to villin (1:2000; clone 1D2C3; Abcam, Cambridge, UK) diluted in PBS-TE (PBS containing 0.1% v/v Triton X-100 and 0.1 mM EDTA). Blocking solution consisted of PBS-TE containing 5% w/v skimmed milk powder. Incubation and washing were carried out using the buffer PBS-TE. Horseradish peroxidase-linked anti-mouse secondary antibody (DAKO Ltd, Cambridge, UK) diluted 1:2000 in PBS-TE was then used, and immunoblots were visualized as described above. Densitometric analysis of Western blots was used to determine relative protein abundance.

Immunohistochemistry

Immunohistochemistry was performed on equine colonic tissue sections based on procedures described previously (Dyer *et al.* 2009). Equine colonic tissue sections, fixed for 4 h in 4% (w/v) paraformaldehyde in PBS, were paraffin wax embedded and sectioned at a thickness of 5–7 μm onto poly-L-lysine-coated slides. The slides were dewaxed in 100% xylene three times, each for 10 min, and then hydrated by stepwise washing in 100% ethanol two times, each for 10 min, 70% ethanol two times, each for 5 min, and double distilled H_2O two times, each for 5 min. Slides were then immersed in antigen retrieval buffer (1 M Tris–HCl, pH 10) and autoclaved two times, each for 15 min, at 121°C and 130.4 kPa. Subsequently, the slides were washed three times, each for 5 min, in PBS followed by 1 h incubation in blocking solution (PBS containing 3% bovine serum albumin, 0.1% NaN_3 and 2% donkey serum) at 25°C in a humidified chamber. Sections were then incubated overnight at 4°C with primary antibodies against either MCT1 or villin, both raised in rabbits (diluted 1:200). To show antibody specificity, sections were also incubated with MCT1 antibody after pre-incubation with 0.5 μg ml^{-1} of the peptide antigen to which the MCT1 antibody was raised. The slides were then washed five times in PBS for 5 min and incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:500. Finally, the slides were washed five times with PBS for 5 min and mounted in Vectashield hard set mounting media with 4′,6-diaminido-2-phenylindole (DAPI; Vector Laboratories, Burlington, CA, USA). Immunostaining was visualized using an epifluorescence microscope (Nikon, Kingston upon Thames, UK), and images were captured with a Hamamatsu digital camera (C4742-96-12G04; Hamamatsu Photonics KK, Hamamatsu City, Japan).

Transport studies

The uptake of [^{14}C]-butyrate into LMVs was assessed at 38°C using a rapid filtration stop technique as described by Shirazi-Beechey *et al.* (1990) and Ritzhaupt *et al.* (1998*a*). The assay was started by the addition of 100 μl of incubation buffer to LMVs (100 μg protein per assay). The composition of the buffers used varied according to the conditions tested. The specific conditions are given in figure legends. After a designated period of time, the reaction was stopped by addition of 5 ml of ice-cold stop solution (100 mM mannitol, 100 mM sodium gluconate and 20 mM Hepes–Tris, pH 7.5). A 0.9 ml portion of the reaction mixture was then removed and filtered under vacuum through a 0.22-μm-pore size cellulose acetate–nitrate filter (GSTF02500; Millipore, Watford, UK). The filter was washed five times for approx. 3 s each time with ice-cold stop solution and placed in a vial containing 4 ml of scintillation fluid (Scintisafe 3; Fisher Scientific). The radioactivity retained on the filter was measured using an LS 6500 multipurpose scintillation counter (Beckmann-Coulter, High Wycombe, UK). All uptakes were measured in triplicate.

For kinetic analyses, the concentration of butyrate in the incubation medium was varied from 0.5 to 50 mM. For studies using inhibitors of MCT1, stock solutions of phloretin (50 mM in methanol), 4-hydroxy cinnamate (4CHC; 100 mM in DMSO), and para-chloromercuribenzoate Na^+ salt (pCMB; 10 mM in vesicle suspension buffer consisting of 100 mM mannitol, 100 mM $NaHCO_3$, 0.1 mM $MgSO_4$ and 20 mM Hepes–Tris, pH 7.5, heated at 37°C to speed up dissolution) were prepared. Luminal membrane vesicles (10 μl = 100 μg protein per assay) were pre-incubated with either 1 μl alcohol/DMSO (control), phloretin (final concentration 0.5 mM), pCMB or 4CHC, (1 mM) for 30 min at 4°C and subsequently used for uptake studies.

Statistics

Comparisons in the enrichment of marker enzymes in LMV preparations and the effect of intravesicular anion on butyrate uptake were determined by Student's unpaired *t* test. The comparisons of the remaining conditions affecting butyrate transport studies were determined using a one-way ANOVA and Dunnett's multiple comparison

Table 1. Enzyme marker activities

Enzyme	Specific activity (nmol min^{-1} (mg protein)$^{-1}$) Homogenate	LMVs	Enrichment*	Recovery† (%)
Cysteine-sensitive alkaline phosphatase	30.5 ± 1.3	225 ± 13	7 ± 0.9	10 ± 1.75
Na^+–K^+-ATPase	0.08 ± 0.04	0.09 ± 0.005	—	1.6 ± 0.5

Abbreviation: LMVs, luminal membrane vesicles. *Specific activity in LMVs/specific activity in the homogenate. †Total activity (in nanomoles per minute) in LMVs as a percentage of the total activity in the homogenate.

post hoc test. All statistical tests were carried out using commercially available software (Graphpad Prism 6 GraphPad Software Inc, La Jolla, CA, USA.).

Results

Characterization of membrane vesicles

In order to determine the membrane origin of isolated vesicles, we determined the activity and abundance of luminal and basolateral membrane marker proteins in the same populations of membrane vesicles and respective cellular homogenates. The specific activity of cysteine sensitive alkaline phosphatase was enhanced sevenfold ($P = 0.0002$) in the vesicles in comparison to the original homogenate with recovery of 10 ± 1.75% (Table 1). Conversely, the activity of Na^+–K^+-ATPase was negligible in the final membrane vesicle suspension (Table 1). Moreover, Western blot analysis for the known markers of luminal membranes, villin (West *et al.* 1988; Pinto *et al.* 1999), and basolateral membranes, GLUT2 (Pinches *et al.* 1993; Dyer *et al.* 2009), demonstrated ninefold ($P = 0.0003$) enrichment of the 95 kDa villin protein and disenrichment of the 50 kDa GLUT2 protein in the final LMVs in comparison to the original cellular homogenates (Fig. 1*A* and *B*).

Expression of MCT1 and SMCT1 by Western blot analysis

Having demonstrated that membrane vesicles originate from the luminal membrane of equine colonic absorptive epithelial cells, we next determined MCT1 and SMCT1 protein expression in the same populations of LMVs and corresponding cellular homogenates. The antibody to MCT1 is well characterized in our laboratory (Cuff *et al.* 2002; Lambert *et al.* 2002). However, in order to determine the reliability of the commercial antibody to SMCT1, postnuclear membranes isolated from human colonic biopsies were used as a positive control, because it has been shown that human colon expresses SMCT1 on the luminal membrane (Gopal *et al.* 2007). By Western blot analysis, we found that MCT1 protein was 14-fold

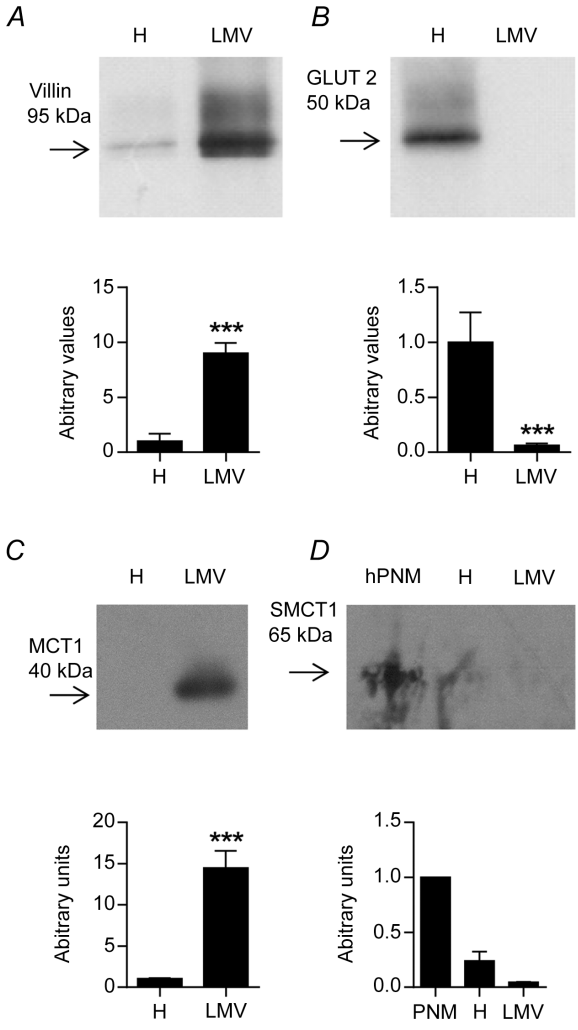

Figure 1. Immunodetection of marker proteins in luminal membrane vesicles (LMVs) isolated from equine colon
Protein components of LMVs, and corresponding cellular homogenates (H; 20 μg protein per lane) were separated on 8% polyacrylamide gel, electro-transferred to polyvinylidene difluoride membranes and probed with antibodies to villin (*A*), glucose transporter isoform 2 (GLUT2; *B*), monocarboxylate transporter 1 (MCT1; *C*) and Na^+-coupled monocarboxylate cotransporter 1 (SMCT1; *D*). In *D*, human colonic postnuclear membranes (hPNM) were used as a positive control for SMCT1. Statistically significant results were determined using Student's unpaired *t* test, where $^{***}P < 0.001$.

($P = 0.0004$) enriched in LMVs in comparison to the original cellular homogenate (Fig. 1*C*). The commercial antibody to SMCT1 reacted with a protein of 65 kDa in human colonic postnuclear membranes and equine colonic cellular homogenates. However, the abundance of this protein was fourfold lower in equine colonic cellular homogenate, and it was hardly detected in the equine colonic LMVs (Fig. 1*D*). The data indicate that SMCT1 is not significantly expressed in the equine colonic luminal membrane (see section *Effect of intravesicular anion on butyrate uptake* for further evidence for the absence of SMCT1).

Immunohistochemical localization of MCT1 protein in equine colon

By immunohistochemistry, we showed that villin, a classical marker of the luminal membrane of colonic epithelial absorptive cells (West *et al.* 1988; Pinto *et al.* 1999), was expressed exclusively on the luminal membrane of absorptive epithelial cells in the horse colon (Fig. 2*A*). Furthermore, the antibody to MCT1 reacted with a protein located entirely on the equine colonic luminal membrane, with a profile similar to that of villin (Fig. 2*B*), supporting the luminal location of MCT1 in equine colon. Notably, this labelling was blocked by pre-incubation of the MCT1 antibody with its corresponding peptide antigen, supporting the specificity of the MCT1 antibody (Fig. 2*C*).

Effect of intravesicular anion on butyrate uptake

It has been shown by Garcia *et al.* (1994*a*,1994*b*) that L-lactate uptake is enhanced severalfold following preloading of either human breast cancer cells or Chinese hamster ovary cells with anions (Garcia *et al.* 1994*a*,1994*b*); therefore, experiments were designed to evaluate the effects of an intravesicular anion on butyrate uptake into equine colonic LMVs. In the presence of pH gradient (pH_o 5.5, pH_i 7.4), preloading the vesicles with bicarbonate increased the rate of butyrate uptake by 12-fold over that measured into mannitol-loaded vesicles (Fig. 3). The uptake was insensitive to the classical anion exchange inhibitors, the sulfonic stilbene derivatives 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and 4,4′- diisothiocyanostilbene-2,2′ disulphonate (DIDS) (0.5 mM; Salhany, 1996; Obrador *et al.* 1998). The transport of butyrate was also shown to be Na^+ independent, because the replacement of Na^+ with K^+ in the incubation medium had no effect on the rate of butyrate uptake. The data suggest that butyrate transport across the colonic LMVs does not involve a classical anion exchanger or a Na^+-dependent mechanism.

Time course of butyrate uptake

The time course of butyrate uptake into bicarbonate-loaded colonic LMVs was measured in the presence of a pH gradient (pH_o 5.5, pH_i 7.4). Figure 4 demonstrates that there was a concentrative accumulation of butyrate or 'overshoot' after 1 min, which was ~10-fold higher than the level measured at the equilibrium point (30 min), suggesting H^+-energized uptake. The rate of transport was linear over the first 5 s, so this time point was used in all subsequent uptake measurements.

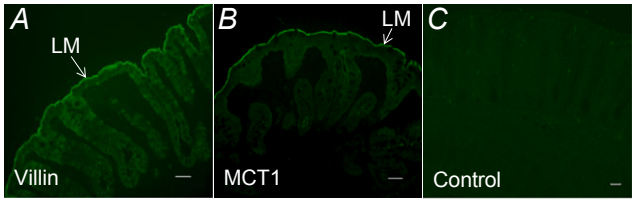

Figure 2. Immunohistochemical localization of MCT1 and villin in equine colon
Sections of horse colonic tissues were immunostained with antibodies to villin (*A*) or MCT1 (*B*; see arrows). Abbreviation: LM, luminal membrane. The MCT1 labelling was blocked by pre-incubation of the MCT1 antibody with the corresponding peptide antigen (*C*). Scale bars represent 10 μm.

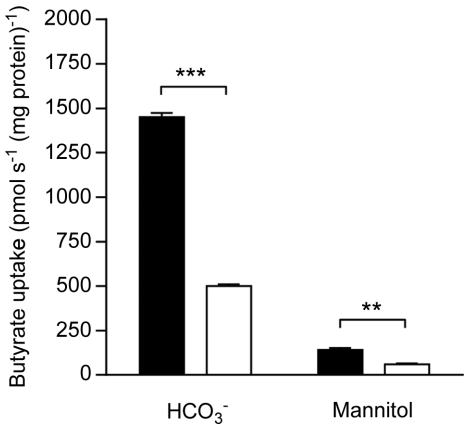

Figure 3. Effect of intravesicular anion on butyrate uptake
Luminal membrane vesicles were loaded with isosmolar solutions of 20 mM Hepes–Tris (pH 7.5), 0.1 mM $MgSO_4$ and either 300 mM mannitol or 100 mM mannitol and 100 mM $NaHCO_3$. Uptake was measured by incubating vesicles (100 μg protein per assay) at 38°C for 5 s in a medium containing 100 mM mannitol, 100 mM sodium gluconate, 0.1 mM $MgSO_4$, 1 mM [^{14}C]-butyrate and either 20 mM Mes–Tris (pH 5.5; filled columns) or 20 mM Hepes–Tris (pH 7.5; open columns). Data were generated in triplicate. Results are shown as means + SEM; $n = 6$. Statistically significant results were determined using Student's unpaired *t* test, where $^{**}P < 0.01$ and $^{***}P < 0.001$.

Effect of varying extravesicular pH on butyrate transport

Given that transport of butyrate via MCT1 is energized by a pH gradient (extravesicular < intravesicular), experiments were conducted to evaluate the effect of varying the pH of the extravesicular medium on the initial rate of butyrate uptake. Equine colonic LMVs preloaded with a buffer containing sodium bicarbonate, pH 7.4 (loading buffer; see legend to Fig. 5) were incubated in isosmolar solutions of varying pH (5.5–8.0). The results presented in Fig. 5 show that there was a six-fold ($P < 0.001$) enhancement of butyrate uptake at pH 5.5 compared with pH 8.0. These results indicate that butyrate transport is energized by a pH gradient ($pH_o < pH_i$).

Kinetics of butyrate transport

In order to examine further the presence of a carrier-mediated transport mechanism for butyrate transport across the equine colonic LMVs, the kinetics of butyrate transport was measured. The initial rate of butyrate uptake was measured with increasing substrate concentration (from 0.5 to 50 mM) in the presence of an inwardly directed pH gradient (pH_o 5.5, pH_i 7.5). Butyrate uptake was saturable and conformed to Michaelis–Menton kinetics (Fig. 6) with an apparent K_m of 5.6 ± 0.45 mM and a maximal velocity of 614 ± 55 pmol s^{-1} (mg protein)$^{-1}$, as determined by linear regression analysis of a Hanes plot (Fig. 6, inset). Linear regression analysis produced a single line ($r^2 = 0.99$), indicating a single transport system (Fig. 6, inset).

Effect of cations and anions on butyrate uptake

Replacing sodium with potassium in the incubation medium had no effect on the initial rate of butyrate uptake into colonic LMVs, excluding the participation of a Na^+-dependent mechanism (Fig. 7). Inclusion of anions of different membrane permeability NO_3^- > Cl^- > SO_4^{2-} > gluconate (Kimmich *et al.* 1985; Shirazi-Beechey *et al.* 1990) in the incubation medium did not influence the initial rate of butyrate uptake into colonic LMVs, suggesting that butyrate transport is not electrogenic (Fig. 7). Gluconate is a membrane-impermeable anion (Kimmich *et al.* 1985; Shirazi-Beechey *et al.* 1990).

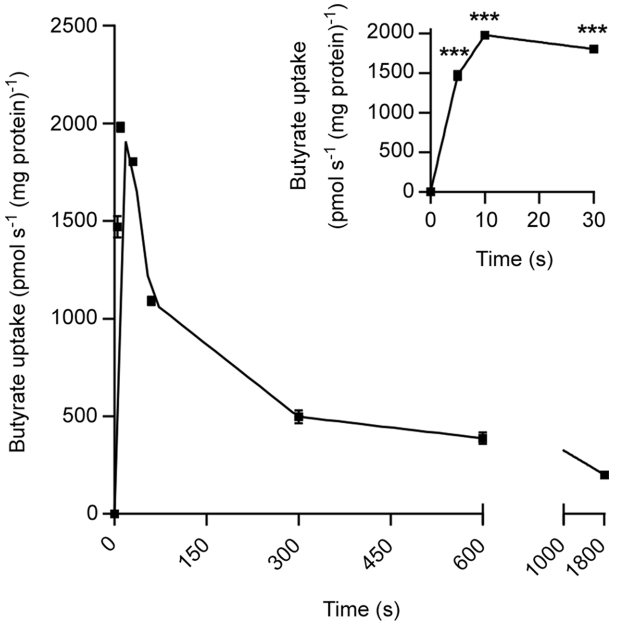

Figure 4. Time course of butyrate uptake
Luminal membrane vesicles were loaded with 20 mM Hepes–Tris (pH 7.5), 0.1 mM $MgSO_4$, 100 mM mannitol and 100 mM $NaHCO_3$. Uptake was measured by incubating vesicles (100 μg protein per assay) at 38°C for a period of time (as indicated on the *x*-axis) in media containing 100 mM mannitol, 100 mM sodium gluconate, 0.1 mM $MgSO_4$, 1 mM [^{14}C]-butyrate and 20 mM Mes–Tris (pH 5.5). The inset shows the initial rate of butyrate uptake. Data were generated in triplicate. Results are shown as means ± SEM; $n = 6$. Statistically significant results were determined using one-way ANOVA with Dunnett's multiple comparison *post hoc* test, where $^{***}P < 0.001$.

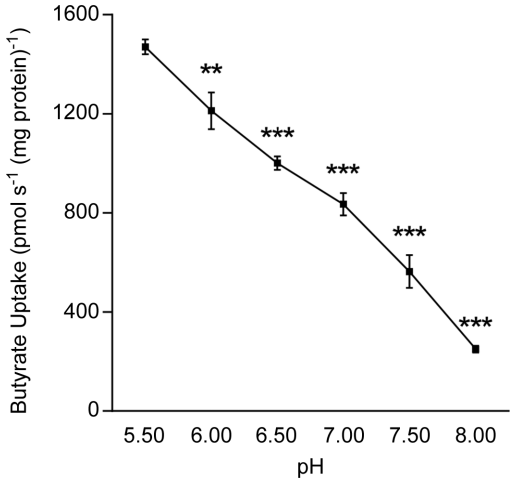

Figure 5. Effect of decreasing extravesicular pH on butyrate uptake
Equine colonic LMVs loaded with 100 mM mannitol, 100 mM $NaHCO_3$, 0.1 mM $MgSO_4$ and 20 mM Hepes–Tris (pH 7.5; 100 μg protein per assay) were incubated at 38°C for 5 s in a medium containing 100 mM mannitol, 100 mM sodium gluconate, 0.1 mM $MgSO_4$, 1 mM [^{14}C]-butyrate and 20 mM Mes–Tris (pH range of 5.5, 6.0, 6.5) or 20 mM Hepes–Tris (pH range of 7.0, 7.5, 8.0). Data were generated in triplicate. Results are shown as means ± SEM; $n = 6$. Statistically significant results were determined using one-way ANOVA with Dunnett's multiple comparison *post hoc* test, (results compared with butyrate uptake at pH 5.5), where $^{**}P < 0.01$ and $^{***}P < 0.001$.

Effect of monocarboxylates on butyrate uptake

Acetate, propionate and butyrate are the main anions present in colonic lumen. In equine colon, the lactate concentration is normally low (Daly *et al.* 2012). We sought to determine effects of acetate and propionate, as well as the monocarboxylates pyruvate and lactate, on butyrate uptake. As seen in Fig. 8, with 1 mM butyrate present in the incubation medium, acetate, propionate and pyruvate at 20 mM each inhibited butyrate uptake by up to 60%. Lactate (20 mM) also inhibited butyrate uptake (by 67%), whereas formate had no effect (Fig. 8).

Effect of MCT1 inhibitors

In order to provide further support for the proposition that butyrate uptake into equine colonic LMVs is via MCT1, the effects of phloretin, 4CHC and pCMB, potent inhibitors of MCT1 (Poole & Halestrap, 1993; Ritzhaupt *et al.* 1998*a*; Gonçalves *et al.* 2009), on the initial rate of butyrate uptake were determined. All three compounds significantly inhibited butyrate uptake by >50% (Fig. 9).

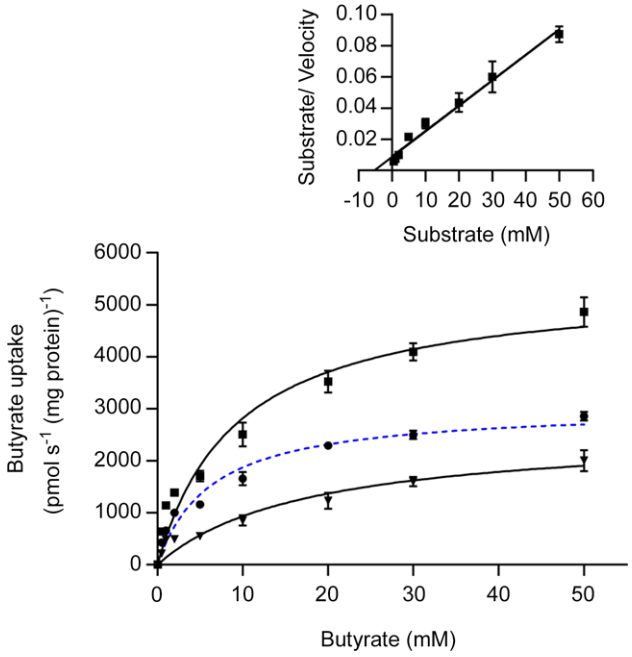

Figure 6. Kinetics of butyrate transport
Initial rates (5 s) of [^{14}C]-butyrate uptake with increasing butyrate concentrations over the range 0.5–50 mM were determined at 38°C. Luminal membrane vesicles (100 μg protein per assay) loaded with 100 mM mannitol, 100 mM $NaHCO_3$, 0.1 mM $MgSO_4$ and 20 mM Hepes–Tris (pH 7.5) were incubated in media containing 100 mM mannitol, 20 mM Mes–Tris (pH 5.5), 1 mM [^{14}C]-butyrate, 0.1 mM $MgSO_4$ and varying concentrations of sodium butyrate and sodium gluconate (to maintain constant medium osmolarity). Carrier-mediated transport (filled circles) was determined by subtracting the uptake values in the presence of a pH gradient (out 5.5, in 7.5; filled squares) from those in the absence of a pH gradient (out 7.5, in 7.5; filled inverted triangles). The inset shows a Hanes plot of linear regression analysis. Data were generated in triplicate. Results are shown as means ± SEM; n = 6.

Discussion

The short-chain fatty acids acetate, propionate and butyrate are products of colonic microbial fermentation of dietary plant fibre. They are absorbed in the intestine and serve as an important source of energy for the horse (Bergman, 1990). Among SCFAs, butyrate exerts potent effects on a variety of colonic functions and plays a key role in the maintenance of the health of colonic tissue (Bugaut & Bentéjac, 1993; Treem *et al.* 1994; Cuff *et al.* 2005; Hamer *et al.* 2008).

Thus far, several studies have been undertaken to examine the mechanism of butyrate transport across the luminal membrane of human, rabbit, pig and rat intestinal tissues (Engelhardt *et al.* 1989; Mascolo *et al.* 1991; Ritzhaupt *et al.* 1998*a*; Sellin & DeSoignie, 1990). The proposed mechanisms include passive non-ionic diffusion of the undissociated form of SCFA and specific carrier-mediated transport of SCFA anions. Suggested mechanisms for carrier-mediated transport of the ionized form of butyrate into colonic epithelial cells include the following: (i) $SCFA^-$–HCO_3^- exchanger

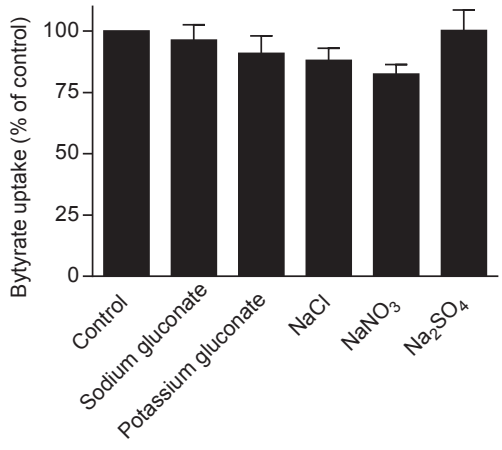

Figure 7. Effect of cations and anions on butyrate uptake
Equine colonic LMVs were preloaded as described in Fig. 6. Luminal membrane vesicles were incubated for 5 s in media containing 100 mM mannitol, 1 mM [^{14}C]-butyrate, 0.1 mM $MgSO_4$, 20 mM Mes–Tris (pH 5.5) and 100 mM of sodium gluconate, potassium gluconate, NaCl or $NaNO_3$ or 67 mM Na_2SO_4 to maintain constant osmolarity. Data were generated in triplicate. Results are shown as the mean percentage of the control value + SEM; n = 6. Statistically significant results were determined using one-way ANOVA with Dunnett's multiple comparison *post hoc* test.
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(Harig *et al.* 1996; Mascolo *et al.* 1991); (ii) an electroneutral H^+-coupled monocarboxylate transporter (MCT1; Hadjiagapiou *et al.* 2000; Stein *et al.* 2000; Cuff *et al.* 2002; Martín-Venegas *et al.* 2007; Lecona *et al.* 2008; Gonçalves *et al.* 2009); and (iii) an electrogenic Na^+-coupled transporter for monocarboxylates (SMCT1; Miyauchi *et al.* 2004).

The mechanism by which butyrate is transported across the equine colonic luminal membrane is unknown. The normal equine colonic luminal pH of ∼7.5 is essential for the maintenance of optimal microbial fermentation of dietary plant fibre (Julliand *et al.* 2001; Daly *et al.* 2012). At this pH, SCFAs (pKa ≈ 4.8) exist predominantly in their anionic forms, the transport of which across the plasma membrane requires a carrier-mediated mechanism.

Here, we report isolation and characterization of luminal membrane vesicles from equine colon using a well-established technique in our laboratory. We have demonstrated by Western blotting and enzyme analyses that the LMVs are enriched in villin and cysteine-sensitive alkaline phosphatase, markers of the luminal membrane, supporting their luminal membrane origin. Furthermore, we showed that LMVs are devoid of Na^+–K^+-ATPase and GLUT2, markers of the basolateral membrane. Moreover, we demonstrated by Western blotting that the LMVs are also enriched in MCT1 in comparison to the original cellular homogenate and do not express SMCT1 protein. Additionally, by immunohistochemistry, we showed that MCT1 protein is exclusively expressed on the luminal membrane of equine colonic absorptive epithelial cells.

Here, we have shown that butyrate is transported into equine colonic LMVs by an electroneutral, H^+-symport mechanism. It has previously been shown that adjacent to the luminal surface membrane of the intestine there is an area of low pH or 'acid microclimate' (Lucas *et al.* 1975; Shimada & Hoshi, 1988), which provides the H^+ electrochemical gradient that acts as the driving force for the absorption of many solutes, such as di/tripeptides, folate, amino acids, organic anions and SCFAs (Anderson & Thwaites, 2010). The majority of proton-coupled transporters, including MCT1, function in association with Na^+–H^+ antiporters (NHE1) involved in intracellular pH and volume regulation (Zachos *et al.* 2005; Thwaites & Anderson, 2007).

In the present study, the kinetic analysis of butyrate uptake into equine colonic LMVs confirmed the presence

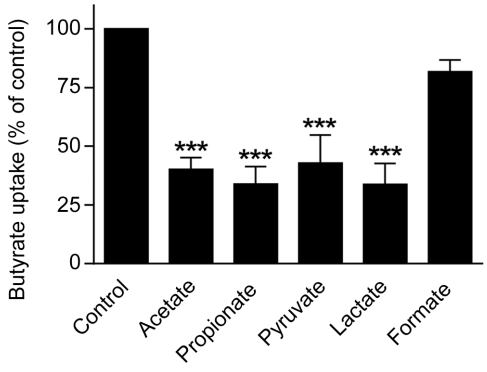

Figure 8. Effect of various monocarboxylates on butyrate uptake
Luminal membrane vesicles (100 μg protein per assay) preloaded as described in Fig. 6 were incubated for 5 s in media containing 100 mM mannitol, 100 mM sodium gluconate, 0.1 mM $MgSO_4$, 1 mM [^{14}C]-butyrate, 20 mM Mes–Tris (pH 5.5), alone or with 80 mM sodium gluconate (rather than 100 mM to maintain a constant osmolarity) and 20 mM of acetate, propionate, pyruvate, lactate or formate. Data were generated in triplicate. Results are shown as the mean percentage of the control value + SEM; $n = 6$. Statistically significant results were determined using one-way ANOVA with Dunnett's multiple comparison *post hoc* test, where $^{***}P < 0.001$.

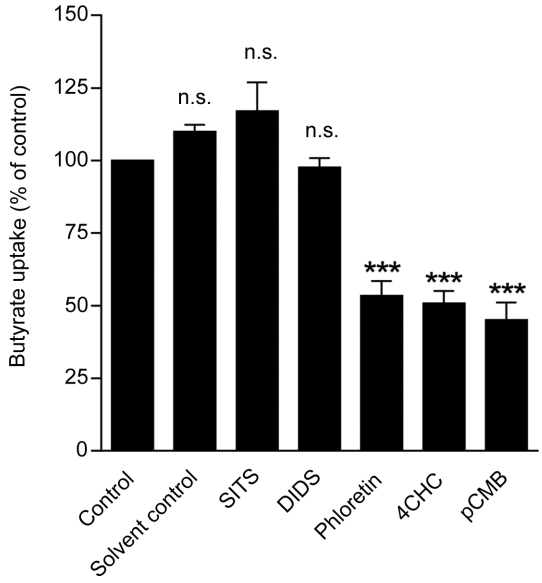

Figure 9. Effect of SITS, DIDS and MCT1 inhibitors on butyrate uptake
Luminal membrane vesicles (100 μg protein per assay) preloaded as described in Fig. 6 were incubated for 30 min at 4°C with either an anion exchange inhibitor [SITS or DIDS (0.5 mM)] or an MCT1 inhibitor [phloretin (0.5 mM), 4CHC (1 mM) or pCMB (1 mM)]. Stock solutions of phloretin, 4CHC and pCMB were prepared by dissolving the compounds in methanol, DMSO or the vesicle suspension buffer, respectively. Luminal membrane vesicles (solvent control) were also incubated with methanol or DMSO and maintained for 30 min at 4°C. Luminal membrane vesicles were then incubated for 5 s in media containing 100 mM mannitol, 100 mM sodium gluconate, 0.1 mM $MgSO_4$, 1 mM [^{14}C]-butyrate and 20 mM Mes–Tris (pH 5.5). Data were generated in triplicate. Results are shown as the mean percentage of the control value + SEM; $n = 6$. Statistically significant results were determined using one-way ANOVA with Dunnett's multiple comparison *post hoc* test, where $^{***}P < 0.001$.

of a single saturable transport system. Butyrate transport was not affected by the inhibitors of anion exchangers, SITS and DIDS, but the classical MCT1 antagonists, phloretin, 4CHC and pCMB, significantly inhibited butyrate uptake. It is noteworthy that impairment of butyrate transport by 4CHC and pCMB but not by SITS and DIDS has previously been reported as a characteristic feature of MCT1 (Ritzhaupt *et al.* 1998*a*; Halestrap & Price, 1999; Hadjiagapiou *et al.* 2000; Lam *et al.* 2010).

The underlying mechanism by which intracellular anion (bicarbonate) enhances the rate of MCT1-mediated transport is not known. However, Garcia *et al.* (1994*a*,1994*b*), who first identified the structure of MCT1 at the molecular level, also showed that L-lactate uptake was enhanced severalfold following preloading of Chinese hamster ovary cells with anions. It is possible that this may be due to intracellular ionic strength.

Moreover, the SCFAs acetate and propionate and the monocarboxylates pyruvate and lactate all inhibited butyrate transport. The lactate/pyruvate inhibition of butyrate transport can be interpreted as either competition for the same binding site as butyrate on the transporter (competitive inhibition) or, alternatively, sharing the same transport protein as butyrate. In the latter case, however, when there is significant accumulation of lactic acid in the intestine of the horse due to particular conditions, the rate of production of lactic acid may far exceed the capacity of MCT1 to absorb lactate.

Any increase in the intraluminal concentration of lactic acid in the colon is of major concern in the horse. It has been shown that in horses given high levels of grain diets (Daly *et al.* 2012) or those suffering from simple colonic obstruction and distension, a prevalent form of dietary-induced intestinal disease, there is a progressive and significant increase in lactic acid-producing bacteria and corresponding lactic acid concentrations (Daly *et al.* 2012). Members of lactic acid-producing species proliferate in starch-rich environments, producing excessive amounts of lactic acid and CO_2 (Goad *et al.* 1998; Goodson *et al.* 1998; Daly *et al.* 2012). Members of this group, notably *Streptococcus bovis*, have long been implicated in the aetiology of lactic acidosis both in horses and in ruminants (Rowe *et al.* 1994; Owens *et al.* 1998; Russell & Rychlik, 2001). Furthermore, horses inherently have low numbers of lactate utilizers, which may contribute to the build-up of lactic acid observed in horses fed high-grain diets (Daly *et al.* 2012). Increased lactic acid, with a subsequent decrease in luminal pH, is likely to suppress the growth of obligate fibrolytic, acid-intolerant bacteria (Miyazaki *et al.* 1992; Asanuma *et al.* 1997), thereby leading to a reduction in fermentation of dietary fibre to SCFAs.

In this study, we have demonstrated that butyrate is transported across the equine colonic luminal membrane by MCT1. It has been shown that expression of intestinal MCT1 and butyrate transport are upregulated by increased luminal concentrations of butyrate in human intestinal cell lines (Cuff *et al.* 2002) and in pig intestine (Haenen *et al.* 2013). Moreover, it has been documented that consumption of some fructan-based prebiotics or resistant starches promotes butyrate-producing bacteria and butyrate production in human (Scott *et al.* 2014) and pig colon (Haenen *et al.* 2013). All together, these findings provide the basis for rational formulation of diets designed to enhance and maintain butyrate-producing bacterial populations in equine colon. This has the potential of increasing butyrate production, upregulation of MCT1 expression and enhancement in butyrate absorption, ensuring the health of the colonic tissue and the horse.

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Additional Information

Competing interests

None declared.

Author contributions

T.N., A.W.M., M.A.A.-R. and S.P.S.-B. designed the research. T.N., A.W.M. and M.A.A.-R. performed the research. S.P.S.-B., T.N., A.W.M. and M.A.A.-R. analysed the data. S.P.S.-B. wrote the paper. All authors approved the final version for publication.

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