

## Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease

Kristian Daly<sup>1</sup>, Christopher J. Proudman<sup>2</sup>, Sylvia H. Duncan<sup>3</sup>, Harry J. Flint<sup>3</sup>, Jane Dyer<sup>1</sup> and Soraya P. Shirazi-Beechey<sup>1\*</sup>

<sup>1</sup>*Epithelial Function and Development Group, Department of Cellular and Molecular Physiology, Faculty of Health and Life Sciences, University of Liverpool, Brownlow Hill, Liverpool L69 7ZJ, UK*

<sup>2</sup>*Equine Division, Department of Veterinary Clinical Sciences, University of Liverpool, Leahurst, Neston CH64 7TE, UK*

<sup>3</sup>*Microbial Ecology Group, The Rowett Institute of Nutrition and Health, University of Aberdeen, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, UK*

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### Abstract

We aimed to determine the effects of variations in dietary composition on equine gut microbiota and their fermentation products, and proposed that dietary modifications profoundly affect microbial ecosystems and their metabolites. Bacterial communities within the large intestine of three groups of horses were compared using oligonucleotide-RNA hybridisation methodology. Each group consisting of six horses was maintained on (1) a grass-only diet, (2) a concentrate diet (i.e. supplemented with hydrolysable carbohydrates) and (3) a concentrate diet but horses were affected by simple colonic obstruction and distension (SCOD), a prevalent form of dietary-induced intestinal disease. We show that in response to dietary change and intestinal disease, there is a progressive and significant increase in Lachnospiraceae, the *Bacteroidetes* assemblage and the lactic acid-producing, *Bacillus–Lactobacillus–Streptococcus* (BLS) group. In contrast, there is a corresponding decrease in the proportion of obligate fibrolytic, acid-intolerant bacteria, *Fibrobacter* and Ruminococcaceae. Assessment of monocarboxylic acids indicated that there are significantly higher concentrations of lactic acid in the colonic contents of horses maintained on a concentrate diet and those suffering from SCOD, correlating with the observed increase in the population abundance of the BLS group. However, the population size of the Veillonellaceae (lactate utilisers) remained constant in each study group. The inability of this group to respond to increased lactic acid may be a contributory factor to the build-up of lactic acid observed in horses fed a concentrate diet and those suffering from SCOD.

**Key words:** Equine intestine: Intestinal microbiota: Diet

Horses are trickle feeders possessing a voluminous and elaborate large intestinal tract. They are hindgut fermenters with a complex microbial digestion uniquely adapted to grazing on high-fibre, low-energy fodder. Domestication of the horse has led to this natural pattern of feeding and digestion being disrupted. Consequently, gastrointestinal dysfunction, such as colic, is the single most important cause of mortality among domesticated horses<sup>(1–7)</sup>.

The microbial hydrolysis of dietary plant fibre (grass) within the large intestine leads to the release of soluble sugars, which are subsequently fermented to monocarboxylates, notably acetate, propionate and butyrate, referred to as SCFA. A large proportion (60–70%) of the horse's body energy is provided by SCFA absorbed from the caecum and the colon<sup>(8–10)</sup>. However, to provide further energy for the demands of work

and athletic performance, many horses are fed diets containing readily hydrolysable carbohydrates (hCHO), generally in the form of grain (starch); commonly referred to as concentrate diets. There is strong epidemiological evidence for the feeding of hCHO as a risk factor for equine intestinal dysfunction<sup>(7)</sup>. This includes evidence of a dose–response relationship between the amount of hCHO and the risk of disease<sup>(3,6,11)</sup>. It is suggested that when horses are introduced to diets containing high levels of hCHO (>0.4% of body weight), a substantial proportion of starch escapes hydrolysis in the small intestine. This reaches the large intestine<sup>(12–14)</sup>, where it is fermented to products such as lactic acid and CO<sub>2</sub>. It has been demonstrated that there is a significant increase in lactic acid concentration in the colon of horses fed high-grain diets<sup>(15)</sup>. This is accompanied by a significant

**Abbreviations:** BLS, *Bacillus–Lactobacillus–Streptococcus*; CFB, *Cytophaga–Flexibacter–Bacteroides*; hCHO, hydrolysable carbohydrates; rRNA, ribosomal RNA; SCOD, simple colonic obstruction and distension; SSU, small subunit.

\* **Corresponding author:** Professor S. P. Shirazi-Beechey, email spsb@liverpool.ac.uk

decrease in caecal/colonic pH (from  $>7.5$  to  $<6.2$ )<sup>(15,16)</sup>, leading to perturbation in the microbiota<sup>(1,17)</sup> disposing the horse to intestinal dysfunction. However, little information is available on the precise changes that may occur in these microbial ecosystems and their underlying mechanisms.

We previously investigated the molecular diversity of the microbiota in the large intestine of horses maintained on pasture forage by the analysis of PCR-amplified small subunit (SSU) ribosomal RNA (rRNA) gene sequences<sup>(18,19)</sup>. We showed that the majority of the recovered sequences are phylogenetically affiliated to several known groups of anaerobic bacteria, namely the low %G+C Gram-positive bacteria belonging to the phylum *Firmicutes*, especially the Lachnospiraceae (cluster XIVa of the Clostridiaceae), and the phylum *Bacteroidetes*, previously known as the *Cytophaga–Flexibacter–Bacteroides* (CFB) phylum. Other notable members included the *Fibrobacter*, the Ruminococcaceae and the *Bacillus–Lactobacillus–Streptococcus* (BLS) group of *Firmicutes*. These studies revealed that the Lachnospiraceae, the *Bacteroidetes* and the Spirochaetaceae make up the largest proportion of bacteria in the equine hindgut.

In the present study, we have used oligonucleotide–RNA hybridisation methodology to characterise and compare the abundance of these predominant bacterial communities. We have also measured microbial fermentation products in the colonic contents of three groups of horses, maintained on a grass-only diet or a concentrate diet or affected by simple colonic obstruction and distension (SCOD), a prevalent form of dietary-induced intestinal disease.

## Methods

### Collection of equine colonic samples

Samples of large-intestinal contents were obtained from six freshly slaughtered horses from the local abattoir (Nantwich, Cheshire, UK) (Group 1). They had been maintained on pasture forage in the mid-Cheshire area with *ad libitum* access to mature grass comprising mainly of *Lolium perenne* (average nutritional value 8.5 kJ of digestible energy/g DM). Groups 2 and 3 comprised twelve horses in light work and were maintained on concentrate feed rich in sugar and starch, either euthanised for reasons other than intestinal disease, mainly limb injuries (six horses) or undergoing surgery after diagnosis of SCOD (six horses) at the University of Liverpool (Liverpool, UK) Philip Leverhulme Equine Hospital. SCOD is the clinical diagnosis assigned to a number of non-ischæmic diseases of the equine colon (e.g. pelvic flexure impaction, colonic displacement and nephrosplenic entrapment) that result in severe abdominal pain. Surgical treatment is indicated for the successful resolution of some types of SCOD. The precise dietary formulations and individual additives given to horses were not available. However, the core diet for all concentrate-fed horses consisted of a coarse mix balancer (2–2.5 kg/d; 20–25 kJ digestible energy/g), hay/haylage (6 kg/d; 60 kJ digestible energy/g) and barley (1 kg/d; 13 kJ digestible energy/g). A typical coarse mix composition is micronised cereal, grass/alfalfa pellets, molasses, vegetables and herbs.

The time between the last meal and collection of samples was between 12 and 16 h in all cases. The animals sampled for the present study were all client-owned horses undergoing euthanasia or veterinary treatment, and hence outside the scope of the Animals (Scientific Procedures) Act 1986. The samples from client-owned animals undergoing veterinary treatment were obtained with the informed consent of their owners, in accordance with University of Liverpool policies.

The horses were adult (7–12 years old) mares and geldings of various breeds. Euthanasia was either by captive bolt (abattoir) or by the injection of a barbiturate (Philip Leverhulme Equine Hospital). The anaesthetised horses received thiopentane, ketamine and isoflurane. There is no evidence that any of these drugs influence gut microbiota. The horses had not received antibiotics at the time of sampling. Gut contents from the pelvic flexure region of the large intestine were removed promptly (within 10 min), after death or during surgery, placed in cryogenic tubes, frozen in liquid N<sub>2</sub> and subsequently stored at  $-80^{\circ}\text{C}$  until use.

### Extraction of nucleic acid from gut content samples

Nucleic acid was extracted from samples of gut contents using the method outlined by Lin & Stahl<sup>(20)</sup> and as described previously<sup>(18,19)</sup>. Frozen samples were thawed, and approximately 1 g aliquots were transferred to 2 ml screw-cap tubes containing 50  $\mu\text{l}$  of 20% (w/v) SDS, 500  $\mu\text{l}$  phenol equilibrated with a 0.1 M-citrate buffer (pH 4.3) (Sigma-Aldrich Company Limited, Poole, Dorset, UK) and sterile acid-washed glass beads (0.1 mm diameter). The samples were beaten for 2 min at room temperature using a mini beadbeater (Biospec Corporation, Bartlesville, OK, USA; Stratech Scientific, Newmarket, Suffolk, UK) and transferred to a 60°C water bath for 10 min before an additional 1 min of beating. The aqueous supernatant was extracted again with buffer-equilibrated phenol and then an additional three times with phenol–chloroform–isoamylalcohol (25:24:1). The nucleic acid extract was then treated with RNase-free DNase 1 to remove any contaminating genomic DNA, and extracted again with phenol–chloroform–isoamylalcohol (25:24:1). Total nucleic acid (primarily rRNA) was precipitated by the addition of 3 M-sodium acetate (0.1 volumes) and isopropanol (0.7 volumes). The RNA pellets were washed with 70% ethanol and resuspended in sterile RNase-free water before final purification with an equal volume of 13% polyethylene glycol (8000); 1.6 M-NaCl. Purified RNA was then resuspended in sterile RNase-free water and stored at  $-80^{\circ}\text{C}$ . RNA integrity was assessed by agarose gel electrophoresis.

### Quantitative oligonucleotide hybridisation

Oligonucleotide probes, targeted to specific bacterial populations of intestinal ecosystems<sup>(19)</sup>, were 5'-end labelled with <sup>32</sup>P using polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. A tenfold excess of the amount of probe necessary to bind to rRNA applied to the membrane was added to 3  $\mu\text{l}$  of 10  $\times$  kinase buffer, 3  $\mu\text{l}$  of 10 mM-spermidine, 1  $\mu\text{l}$  of polynucleotide kinase (New England Biolabs (UK) Limited, Hitchin, Herts,

UK), an equimolar amount of end-labelling grade [ $\gamma$ - $^{32}$ P] ATP (Perkin Elmer LAS, Beaconsfield, Bucks, UK), and RNase-free water in a total volume of 30  $\mu$ l. The reaction was then incubated at 37°C for 30 min. Unincorporated  $^{32}$ P was removed from labelled probes using NICK columns (GE Healthcare UK Limited, Little Chalfont, Bucks, UK). Extracted RNA at 100  $\mu$ g/ml was denatured by the addition of 2% (v/v) glutaraldehyde and diluted to a final concentration of 4  $\mu$ g/ml with RNase-free water containing 1  $\mu$ g Poly(A)/ml and 0.02  $\mu$ l of 2% (w/v) bromophenol blue/ml. A 100  $\mu$ l volume sample (400 ng) was then applied in triplicate to a positively charged nylon membrane (Hybond N+; GE Healthcare UK Limited) using a slot-blot device under vacuum (Scotlab, Coatbridge, Scotland, UK). Dilution series of known SSU rRNA amounts from appropriate positive controls were included as hybridisation standards on each membrane. The membranes were air dried and rRNA was fixed to the membrane by UV cross-linking (UV Stratalinker 2400; Agilent Technologies, Amsterdam, The Netherlands).

The membranes were prehybridised for 2 h at 40°C in a hybridisation buffer (0.9 M-NaCl, 50 mM-NaH<sub>2</sub>PO<sub>4</sub> (pH 7.0), 5 mM-EDTA, 10  $\times$  Denhardt's solution, 0.5% (w/v) SDS and 0.1 mg Poly(A)/ml) before the addition of 400  $\mu$ l labelled probe in a volume of 10 ml hybridisation buffer. Hybridisation was performed for 16–18 h at 40°C. After hybridisation, the membranes were washed in 1  $\times$  saline-sodium citrate buffer; 1% (w/v) SDS for 15 min at 40°C, followed by washing twice at the appropriate wash temperature for 30 min as described previously<sup>(19)</sup>.

Total SSU rRNA abundance was inferred by the use of a universal hybridisation probe (Univ1390) complementary to all characterised SSU rRNA<sup>(21)</sup>. Following exposure to X-ray film (Kodak MS; Eastman-Kodak, Henel Hempstead, Herts, UK), hybridisation signals were quantified by scanning densitometry using Phoretix 1D Quantifier v.5.10 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). For the quantification of SSU rRNA, standard curves were constructed by linear regression from the known SSU rRNA amounts applied to each membrane. The relative population abundance of specific bacterial groups was then calculated as a fraction of the total SSU rRNA abundance as determined by the hybridisation of the universal probe.

### Measurement of microbial metabolites

Thawed intestinal contents were centrifuged at 13 000 g (Eppendorf 5415C; Eppendorf UK Limited, Cambridge, UK) and the supernatants were removed for the determination of monocarboxylic acid concentration. The method of Richardson *et al.*<sup>(22)</sup> was used to measure the concentrations of naturally occurring monocarboxylic acids (butyric, propionic and acetic) and other analogues (lactic, valeric and isobutyric acids). Briefly, to 1 ml of gut content liquor, 50  $\mu$ l of an internal standard (0.1 M-2-ethyl butyric acid) were added. Monocarboxylic acids were then extracted by the addition of concentrated HCl and diethyl ether. The diethyl ether layer extract was derivatised, and the monocarboxylic acids and derivatives were separated by capillary GC and quantified in relation to the internal standard.

### Statistical analysis

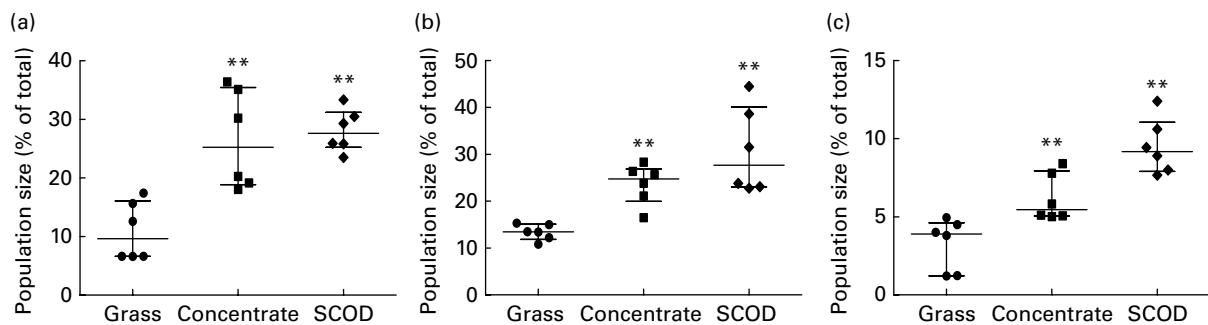
Data in Figs. 1–3 are presented as medians (interquartiles). The points represent individual animals. The significance of differences was determined by using the Mann–Whitney *U* test for non-normally distributed data (GraphPad Prism 5; GraphPad Software, Inc. La Jolla, CA, USA). The results were considered significant if  $P < 0.05$ . *P* values were confirmed by one-way repeated-measures ANOVA with Bonferroni's multiple comparison test.

The data in Table 1 are presented as means and standard errors. The significance of differences was determined using Student's *t* test (GraphPad Prism 5; GraphPad Software, Inc.). The results were considered significant if  $P < 0.05$ . *P* values were confirmed by one-way repeated-measures ANOVA with Bonferroni's multiple comparison test.

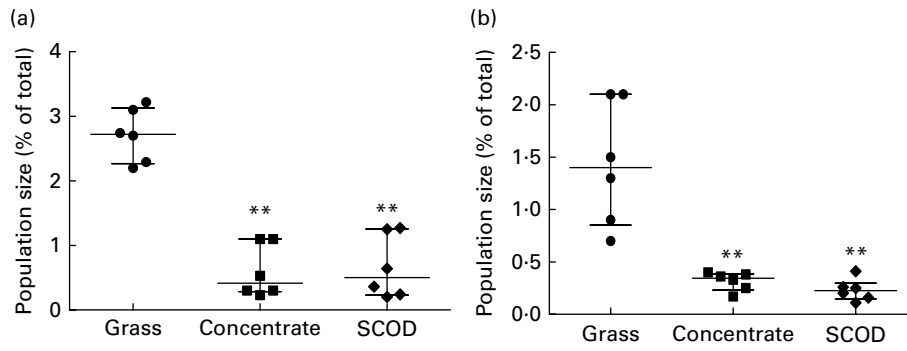
## Results

### Alteration in microbiota in response to dietary change and intestinal dysfunction

To characterise the major microbial populations present in the equine colon, we used oligonucleotide–RNA hybridisation to determine the relative population sizes of the specific



**Fig. 1.** Increased population abundance of the (a) Lachnospiraceae (cluster XIVa of the Clostridiaceae), (b) *Bacteroidetes* assemblage (*Cytophaga*–*Flexibacter*–*Bacteroides*) and (c) *Bacillus*–*Lactobacillus*–*Streptococcus* group in concentrate-fed and simple colonic obstruction and distension (SCOD) horses compared with grass-fed horses, as determined by quantitative oligonucleotide–RNA hybridisation. Data are presented as medians and interquartiles, *n* 6. Points represent individual animals. Significance of differences was determined using the Mann–Whitney *U* test for non-normally distributed data (GraphPad Prism 5; GraphPad Software, Inc.). Results were considered significant if  $P < 0.05$ . *P* values were confirmed by one-way repeated-measures ANOVA with Bonferroni's multiple comparison test. \*\* Values were significantly different from that of the grass-fed group ( $P < 0.01$ ).



**Fig. 2.** Decreased population abundance of (a) *Fibrobacter* spp. and (b) Ruminococcaceae (cluster IV of the Clostridiaceae) in concentrate-fed and simple colonic obstruction and distension (SCOD) horses compared with grass-fed horses, as determined by quantitative oligonucleotide-RNA hybridisation. Data are presented as in Fig. 1. \*\* Values were significantly different from that of the grass-fed group ( $P < 0.01$ ).

microbial communities. Bacterial SSU rRNA, extracted from colonic contents of three different groups of horses, was used in conjunction with seven different group-specific oligonucleotide probes to quantify predominant bacterial populations.

The abundance of Lachnospiraceae (cluster XIVa of the Clostridiaceae) and the *Bacteroidetes* (CFB), targeted by probes EREC482<sup>(23)</sup> and CFB935<sup>(19)</sup>, respectively, were significantly greater in concentrate-fed horses and in concentrate-fed horses suffering from SCOD in comparison with horses maintained on grass diet only.

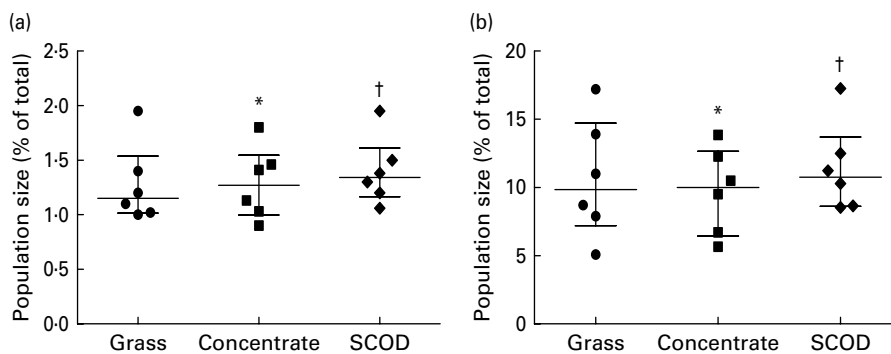
The median population size of the Lachnospiraceae in horses on grass ( $n$  6) was 9.6% of the total microbiota. However, the population size of this group was higher in all concentrate-fed horses and those with SCOD. The median population sizes were greater, with 25.2% in the former ( $n$  6;  $P < 0.01$ ) and 27.6% in the latter group of horses ( $n$  6;  $P < 0.01$ ) (Fig. 1(a)).

The median population size of the *Bacteroidetes* assemblage in grass-fed horses was 13.5% ( $n$  6). This population was 24.7% in concentrate-fed horses ( $n$  6;  $P < 0.01$ ), with all six horses having a significantly higher population. The median population size of the *Bacteroidetes* in horses with SCOD was 27.7% ( $n$  6;  $P < 0.01$ ), with all six horses possessing a significantly higher proportion of *Bacteroidetes* microbes (Fig. 1(b)).

Similar to the Lachnospiraceae group and the *Bacteroidetes* assemblage, there was a higher relative population size of the BLS group in both concentrate-fed and SCOD horses. The median population size, as determined by the BLS1295 probe<sup>(19)</sup>, was 5.5% ( $n$  6;  $P < 0.01$ ) in concentrate-fed horses and 9.2% in horses with SCOD ( $n$  6;  $P < 0.01$ ). This compares with the median population size in grass-fed horses of 3.9% ( $n$  6). All concentrate-fed and SCOD horses contained significantly higher numbers of the BLS group than horses fed on grass only (Fig. 1(c)).

Contrasting effects on other microbial populations were observed in the study groups. The median population size of the *Fibrobacter* group in grass-fed horses was 2.7%, as measured by the FIB225 probe<sup>(24)</sup>. However, in concentrate-fed horses and those with SCOD, *Fibrobacter* comprised 0.4 and 0.5%, respectively ( $n$  6;  $P < 0.01$  for both groups); all concentrate-fed and SCOD horses contained significantly less *Fibrobacter* compared with those given grass (Fig. 2(a)).

Using the IV815 probe<sup>(19)</sup>, we determined the median population size of the Ruminococcaceae (clostridial cluster IV) to be significantly lower in concentrate-fed horses without (0.3%;  $n$  6;  $P < 0.01$ ) and with SCOD (0.2%;  $n$  6;  $P < 0.01$ ) compared with grass-fed horses (1.4%). Again, all concentrate-fed horses and all horses with SCOD had significantly less Ruminococcaceae than those on grass (Fig. 2(b)).



**Fig. 3.** Population abundance of (a) Veillonellaceae (cluster IX of the Clostridiaceae) and (b) the Spirochaetaceae, as determined by quantitative oligonucleotide-RNA hybridisation, showing no significant differences between grass-fed, concentrate-fed and simple colonic obstruction and distension (SCOD) horses. Data are presented as in Fig. 1. (a) Values were not significantly different for concentrate-fed and SCOD horses from that of the grass-fed group, respectively: \*  $P = 0.82$ ; †  $P = 0.38$ . (b) Values were not significantly different for concentrate-fed and SCOD horses from that of the grass-fed group, respectively: \*  $P = 0.82$ ; †  $P = 0.70$ .

**Table 1.** Concentration of monocarboxylic acids in equine colonic contents (Mean values with their standard errors)

Monocarboxylic acid	Concentration (mm)					
	Grass-fed (n 6)		Concentrate-fed (n 6)		SCOD (n 6)	
	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	55.5	2.1	44.7	3.9	50.1	4.2
Propionic acid	11.1	0.9	12.4	1.5	14.0	0.8
Butyric acid	2.9	0.3	3.7	0.5	3.7	0.5
Lactic acid	1.0	0.3	2.5*	0.2	6.0**	1.0
Isobutyric acid	1.3	0.1	1.3	0.1	1.5	0.2
Valeric acid	0.4	0.1	0.6	0.1	0.6	0.1

SCOD, simple colonic obstruction and distension.

Mean values were significantly different from that of the grass-fed group and were determined using Student's *t* test and one-way repeated-measures ANOVA:

\*  $P < 0.05$ ; \*\*  $P < 0.01$ .

The median population level of Veillonellaceae (probably lactate-utilising microbes) in grass-fed horses was 1.2% of total, as determined by the IX854 probe<sup>(19)</sup>. There was no difference in the relative population size of this microbial group in horses that were either maintained on a concentrate diet or had developed SCOD (median population sizes 1.3% for both groups) (Fig. 3(a)).

The Spirochaetaceae is a major component of the equine colonic microbiota, comprising up to 15% of total<sup>(19)</sup>. Use of the Spiro1400 probe in the present study showed that the median population size of this group was similar in all three sets of animals (grass 9.9%; concentrate 10.0%; SCOD 10.8%) (Fig. 3(b)).

### Changes in microbial metabolites

The mean concentration of lactic acid in the colonic contents of horses fed only on grass was 1.0 mm (n 6), with one horse having no detectable lactic acid. However, the mean steady-state concentration of lactic acid in colonic contents of horses consuming concentrate or having developed SCOD were 2.5 mm (n 6;  $P < 0.01$ ) and 6.0 mm (n 6;  $P < 0.01$ ), respectively (Table 1). The concentrations of other monocarboxylic acids (acetic, propionic, butyric, valeric and isobutyric), although variable among individuals, were not significantly different in the intestinal contents of the three groups of horses (Table 1).

### Discussion

In the present study, we show that the population sizes of the Lachnospiraceae, the *Bacteroidetes* (CFB) assemblage and the BLS group are significantly greater in horses fed concentrate and those with the clinical diagnosis of SCOD.

The Lachnospiraceae group is a large and diverse cluster of both fibrolytic and saccharolytic microbes including *Clostridium* spp., *Butyrivibrio* spp., *Ruminococcus* spp. and *Eubacterium* spp. The *Bacteroidetes* assemblage in equine colon is comprised mainly of *Bacteroides* and *Prevotella* spp., which are predominantly saccharolytic and will readily ferment

hCHO<sup>(25)</sup>. Members of both groups possess the ability to express many different genes involved in fibrolytic/saccharolytic digestion and as such can readily adapt to changes in substrate availability<sup>(26,27)</sup>. The enhancement in relative population sizes of these groups is probably a reflection of the increased competitiveness of adaptable members, compared with those that are obligate fibrolytics, in response to the increased amount of hCHO available as substrate.

Members of the BLS, Gram-positive, lactic acid-producing species, favour hCHO as a substrate for fermentation. They do not ferment structural carbohydrates directly but utilise oligosaccharides, released by fibrolytic organisms. They will react rapidly to the availability of more readily fermentable substrates<sup>(28,29)</sup> proliferating in a starch-rich environment producing excess amounts of lactic acid and CO<sub>2</sub><sup>(17,30)</sup>. Members of this group, notably *Streptococcus bovis*, have long been implicated in the aetiology of lactic acidosis both in horses and in ruminants<sup>(28–32)</sup>.

In contrast, *Fibrobacter* spp. and members of the Ruminococcaceae (clostridial cluster IV) are obligate fibrolytic, predominantly acid-intolerant bacteria whose growth is greatly suppressed at acidic pH<sup>(33–36)</sup>. The median proportions of these bacterial populations were significantly lower in horses fed concentrate or undergoing surgery for SCOD, suggesting a more acidic colonic pH. *Fibrobacter* and Ruminococcaceae ferment dietary fibre to SCFA<sup>(37)</sup>. The lower population size of these groups would be expected to result in a reduction in fibre digestion and hence concentrations of intraluminal SCFA. However, our data indicate that the overall concentrations of SCFA remained the same in the intestine of the three study groups of animals. We speculate that this may be due to inhibition of SCFA transport in the intestine of concentrate-fed horses by lactate, a competitive inhibitor of the SCFA transporter, monocarboxylate transporter 1<sup>(38–40)</sup>.

There were significantly higher concentrations of lactic acid in the colonic contents of concentrate-fed horses and those with SCOD compared with grass-fed horses (2.5- and 6-fold higher concentrations, respectively), correlating with the observed greater population abundance of the BLS group. It has been demonstrated that there is a significant increase in lactic acid concentration in the colon of horses fed high-grain diets<sup>(15)</sup>. This is accompanied by a marked decrease in caecal/colonic pH (from  $>7.5$  to  $<6.2$ )<sup>(15,16)</sup>. Lactic acid (as opposed to acetic, butyric or propionic acids) is much more likely to cause drastic alterations in colonic pH due to its relatively low pK<sub>a</sub> of 3.86, thus making it a stronger acid than the other SCFA (the pK<sub>a</sub> of acetic, butyric and propionic acids are 4.76, 4.82 and 4.87, respectively).

Despite the higher lactic acid concentration, the population size of the Veillonellaceae (lactate utilisers; cluster IX of the Clostridiaceae) was similar (approximately 1% of total) in each study group of horses. In contrast, representatives of this group can comprise up to 10% of the total microbial population in the human colon<sup>(41)</sup>. In addition, one important group of Lachnospiraceae found in human colon (related to *Eubacterium hallii* and *Anaerostipes* spp.) has the ability to convert lactate to butyrate<sup>(42)</sup>. Consequently, in humans, most of the lactic acid produced by the fermentation of

resistant starch reaching the colon is converted into butyrate and propionate<sup>(43,44)</sup>. Similarly, in the rumen members of the Veillonellaceae, such as *Selenomonas* spp., often produce lactate, but many strains can convert lactate into propionate<sup>(45)</sup>. However, in horses maintained on grass, the median population size of Veillonellaceae was just over 1% of the total; there was also very little lactic acid present in their colonic contents. The smaller population size of this group in horses may be sufficient when horses are maintained on their natural diet (grass), but an inability to adapt to changes in the colonic environment may contribute to the observed increase in lactic acid.

In order to provide energy for the demands of work and athletic performance, many horses are fed diets containing hCHO, generally in the form of grain (concentrate diet). It is proposed that when they are introduced to diets containing high levels of hCHO (>0.4% of body weight), a substantial proportion of starch reaches the large intestine<sup>(12–14)</sup> causing drastic alteration in microbiota, microbial fermentation products and perturbing caecal and colonic pH<sup>(1,15–17)</sup> disposing the horse to intestinal dysfunction. Horses have low levels of pancreatic  $\alpha$ -amylase, and hence an inability to hydrolyse large amounts of starch, compared with omnivorous species<sup>(46,47)</sup>. It has been shown, in other species, that activity of  $\alpha$ -amylase increases in response to dietary hCHO<sup>(48,49)</sup>. There is evidence that horses are also able to enhance the activity of this enzyme in response to increased dietary hCHO, but this is a lengthy process requiring up to 2–3 weeks<sup>(14)</sup>. Furthermore, the timescale of increased risk of SCOD<sup>(6)</sup> indicates maximum danger during the 7 d following dietary change (hCHO supplementation), with risk declining to baseline levels by 14 d. This is consistent with the time required for up-regulation of intestinal  $\alpha$ -amylase.

Diet has been shown to influence intestinal microbiota resulting in microbial products either beneficial or harmful to the host<sup>(50,51)</sup>. In the present study, we have shown that equine intestinal microbiota are different in horses fed diets varying in hCHO, and that these differences are more exaggerated in a model of dietary-induced intestinal disease. The data demonstrate that differences in diet correlate well with alterations in intestinal microbial ecosystems and production of microbial fermentation products (such as lactic acid), thus creating environments predisposing the host to intestinal diseases.

To further explore associations between dietary change, microbiota and risk of intestinal disease, a case–control study design, where horses of similar age, background and exercise regimen can be managed before dietary intervention, will provide a more direct comparison. A better understanding of molecular mechanisms involved in diet, microbiota and host interactions will allow a more scientifically based approach to designing food formulation, supplementation and management with the ultimate aim of enhancing the health of the species.

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follows: S. P. S.-B. and K. D. designed the study. K. D. performed the study. S. P. S.-B., K. D. and J. D. analysed the data. C. J. P. provided equine colonic samples and dietary/clinical advice. S. H. D. and H. J. F. provided the analytical data on metabolite measurements. S. P. S.-B. and K. D. wrote the manuscript. The authors declare that there is no conflict of interest.

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