Animal (2007), **1**: Pages 367–373 © The Animal Consortium 2007
doi: 10.1017/S1751731107661850

animal

Effect of lactulose on growth performance and intestinal morphology of pre-ruminant calves using a milk replacer containing *Enterococcus faecium*

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(Received 16 May 2006; Accepted 13 November 2006)

The synthetic disaccharide lactulose is known to improve the intestinal microflora by stimulating the growth of selected probiotic bacteria in the gut. In our experiment the effects of lactulose in combination with the probiotic bacteria Enterococcus faecium on growth performance and morphology of the bovine intestine were examined. Calves aged 39 ± 2 days were randomised to three feeding groups (no. = 14 each group): control (L0), fed milk replacer (MR) containing E. faecium*; a lactulose group (L1) containing additional 1% lactulose and a second lactulose group (L3) containing 3% lactulose dry matter. The calves were weighed weekly. After 19 weeks the calves were slaughtered and tissues were collected for histological studies. The average daily live weight gain tended to be higher (*$P < 0.1$*) for L3 (1350 g/day) than L0 (1288 g/day). Compared with L0, a reduction (*$P < 0.001$*) of ileal villus height due to lactulose treatment of approximately 14% in group L1 and 20% in L3 was determined. A significant decrease in the depth of the crypts about 12% in L1 and 8% in L3 was detected in the caecum. The surface area of lymph follicles from Peyer's patches was decreased by lactulose treatment. Results show that lactulose has an effect on the morphology of intestine. A significant effect on growth performance can not be confirmed. However, results permit the conclusion that lactulose feeding has the tendency to increase growth performance.*

Keywords: calves, growth, lactulose, probiotics, villi

Introduction

A growing area of research is the functional effect of probiotics and prebiotics (Hughes and Rowland, 2001). Probiotics are well defined strains of micro-organisms which beneficially affect the host by improving its intestinal microbial balance (Bezkorovainy, 2001). Increased levels of probiotics in the intestine may be achieved by consumption of dietary substrates (i.e. prebiotics) that are known to stimulate probiotic growth (Mosenthin and Zimmermann, 2000). It has been suggested that a combination of pro- and prebiotics, the so-called synbiotics, might be more active than the individual components (Roberfroid, 1998). The knowledge that the normal intestinal flora has a protective function against infection provides the basis for the use of probiotics and prebiotics (Gorbach, 2000; McNaught and MacFie, 2001). The application of this know how in veterinary medicine and its versatile use plays an increasingly important role (Vanbelle *et al.*, 1990), in particular when the protective potential of the microbial gut flora is reduced, for example during stress. The vitality and the well being of the animals can be improved and digestive problems and losses caused by nutrition reduced. Prebiotics and probiotics can improve feed conversion and daily weight gain (Krueger *et al.*, 2002; Busch *et al.*, 2004). Various factors like the early separation from their mother, dietary changes or transportation and the contact with a multiplicity of infectious agents could cause the high incidence of intestinal disease in calves. Hence, animals consume less milk (Loerch and Fluharty, 1999), are predisposed to loss of barrier function of the gut (Nabuurs *et al.*, 2001; Soderholm and Perdue, 2001), and may be afflicted with impaired immune function (Sheridan *et al.*, 1994).

Numerous scientists investigated the health-promoting effect of prebiotics like indigestible sugars, e.g. fructo-oligosaccharides (FOS), inulin and lactulose (Gibson *et al.*, 1995; Kleesen *et al.*, 2001). The positive effects of lactulose

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on colonic metabolism in human, rat, mouse, and pig are well known (Bianchi *et al*., 1997; Ballongue *et al*., 1997). In calves, the effect of lactulose on the intestinal morphology is not investigated in detail. The semisynthetic disaccharide lactulose is chemically well characterised and does not occur naturally. Lactulose cannot be digested by mammalian enzymes because of its specific structure (4-ß-D-galactopyranosyl-D-fructose). It is poorly absorbed from the small intestine and is a suitable substrate for some bacteria in the gut, especially in the colon (Schumann, 2002). *In vitro* investigations demonstrated that lactulose is readily fermented by *Bifidobacteria* and *Lactobacilli*, but also by *Clostridium perfringens, Escherichia coli* and *Bacteroides* sp. (Smart *et al*., 1993). These bacteria counteract detrimental species such as *Clostridia* or *Salmonellae* (Schumann, 2002) which are, like other pathogenic bacteria, not able to digested lactulose (Johnson, 2001).

The objective of this investigation is to determine the influence and effect of a long-term daily lactulose application on the growth performance and the intestinal morphology in growing calves. In addition the influence of two different lactulose concentrations has been investigated. Thus, we have performed histomorphometrical measurements from the small and large intestine to monitor effects on the morphology of the gastro-intestinal tract (GIT) in pre-ruminant calves.

Material and methods

Animals, husbandry, feeding and experimental procedures

Simmental calves were bred at various farms and directly bought from the Simmental breeding organisation (Zuchtverband für oberbayerisches Alpenfleckvieh e.V.) in Miesbach, Germany. The calves were single-born and immediately separated from their mothers after birth. Calves received post-partum colostrum for 1 week directly from their mother cows, as recommended by the breeding organisation. Afterwards until start of the experimental feeding trial, the calves were fed with milk replacer (MR). The 42 calves were divided in three homogenous experimental groups (no. = 14 per group, each 50% male and 50% female) with balanced weight (74.4 (± 2.1) kg) and age (39.0 ± 2.5 days), whereas the females were slightly heavier than males. Animals were housed at the experimental station Karolinenfeld (Bayerische Landesanstalt für Landwirtschaft - LfL, Institut für Tierernährung und Futterwirtschaft) in two segmented pens, half on straw and half on solid floor.

During the feeding experiment, all calves were fed with MR from Milkibeef Top (Milkivit, Trouw Nutrition, Burgheim, Germany) with following composition: 50.2% skimmed milk, 22.5% crude protein (CP), 19.5% crude fat and 10^9 colony forming units (c.f.u.) *Enterococcus faecium* per kg. All calves had free group access to fresh water and 0.5 kg hay per day. Feeding group L0 served as control. The other two groups were fed with MR enriched by 1% (L1) and 3% (L3) dry matter (DM) lactulose (Lactusat, Milei GmbH, Germany). Contents of Lactusat are shown in Table 1, as stated by the manufacturer (Milei). In order to assure the accuracy of the lactulose concentration in the feeding groups L1 and L3 the MR was mixed with 2.5% Lactusat for group L1 and 7.5% Lactusat for group L3. It was exchanged against whey powder to guarantee balanced feeding regimens (Table 2). DM, crude ash, crude fat, starch, CP and calculated metabolisable energy were formulated to be similar across treatments. Calves of all feeding groups received MR in volumes up to 17.5 l/day in the experimental period of 19 weeks (with corresponding amount of lactulose for L1 and L3), controlled by transponder automatic feeder (Förster Technik, Engen, Germany). The MR was reconstituted in hot water (65°C) and fed at a temperature of approximately 41°C. The starting MR concentration at the beginning of the study was 125 g/l (week 1), with a continuous and linear increase up to 250 g/l at the end of the study (week 19). Calves were weighed every week after feeding, before killing and also the empty body weight was measured. After the dosing period of 133.6 ± 8.3 days, animals were slaughtered. The last feeding before slaughtering and tissue sampling was 4 ± 1 h.

Health status

The general health status of the calves was monitored by daily physical examination, checking general appearance, animal activity, faeces composition, and time to time rectal temperature. Animals were further inspected by a veterinarian on a weekly basis to confirm identical healthy status of the feeding groups. The experimental procedures followed the current German law on animal production and veterinary inspection (LfL, Grub, Germany).

Histology and histomorphometry of intestinal mucosa

After slaughtering the GIT was removed and tissue slices of 5 to 7 mm from the small intestine (middle parts of jejunum and ileum) and large intestine (mid caecum, mid colon) were collected. Immediately after collection, the tissue-samples were washed in physiological 0.9% NaCl solution and placed in neutral buffered 10% formalin (Carl Roth GmbH, Karlsruhe, Germany) for 24 h. The specimen

Table 1 *Ingredients of Lactusat (Milei GmbH, Germany)*

Ingredients	%	Ingredients	mg per 100 g
Water	4	Calcium	200
Protein	30	Potassium	250
Ash	1.0	Sodium	150
Fat	<5	Magnesium	30
Lactulose	42	Phosphorus	130
Galactose	3	Chloride	50
Lactose	7	pH	6.4
Epilactose	2		
Fructose	<1		
Tagatose	<1		

https://doi.org/10.1017/S1751731107661850 Published online by Cambridge University Press

Table 2 *Raw nutrient and energy content of diets (the energy content of the milk replacer was estimated by the feeding programme Zifo (LfL, 2005))*

	L0 n = 3	L1 n = 3	L3 n = 3	Pooled s.e.	Hay
Dry matter (DM, g/kg)	964 ± 5	963 ± 6	963 ± 6	6	854
Raw ash (g/kg DM)	70 ± 2	70 ± 3	68 ± 2	2	52
Crude protein (g/kg DM)	219 ± 4	224 ± 1	234 ± 5	5	124
Crude fat (g/kg DM)	197 ± 2	197 ± 2	196 ± 1	1	16
Crude fibre (g/kg DM)	0 ± 0	0 ± 0	0 ± 0	0	311
Metabolisable energy (MJ/kg DM)	16.8 ± 0	16.8 ± 0	16.9 ± 0	0	9.5

were later trimmed and embedded in paraffin. Thin sections (7 to 8 μm) were cut using the Microtom LEICA RM2145 (Leica, Wetzlar, Germany), mounted on glass slides, and stained with haematoxylin and eosin (HE) and covered with Euktit (Merck, Darmstadt, Germany).

Histological sections were examined with the light microscope Axioskop 2 plus (Zeiss, Oberkochen, Germany) with 10 × /0.30 Plan-Neofluar objective connected to the video-based, computer-linked AxioVision 3.1 system that was programmed to perform morphometrical analysis (Blättler *et al*., 2001). Only for the measurement of the lymph follicle the Stemi 2000-C (Zeiss) was used with the × 2.5 objective. Pictures were taken with the AxioCam MRc (Zeiss). The applied objective was changed depend on the examined tissue.

Villus height, crypts depth and for both the width were evaluated on three well orientated villi- and crypt-preparations for each intestinal sampling site. Triplicate measurements for every category (height, depth, width) and section (jejunum, ileum, caecum, colon) were evaluated. Figure 1 illustrates the measurements that were made. Furthermore, the area of at least six lymph follicle in Peyer's patches in the ileum (no. = 84 per group) were evaluated. For confirming the uninjured mucosa integrity of the collected samples, the following qualitative criteria were controlled: villus fusion, villus atrophy, crypt architectural disruption, disruption or distortion of epithel cells and lymph follicles.

Statistical analysis

All measurement values are expressed as mean ± s.e. For group differences, villus heights and width, villus height/width ratios, crypt depths and width and surface of the lymph follicle of Peyer's patches were analysed with the program of Statistical Packages for the Social Sciences (2003) using two-way ANOVA. In order to find out whether lactulose has different sex-specific effects the pairwise multiple comparison procedures were processed with the Holm-Sidak method. The significance level was set at 0.05 for all tests.

Results

All calves stayed healthy and no animal losses were registered during the feeding experiment. No medication was applied to the animals during the 19 weeks.

Feed intake and body weight

The average daily MR intake (Table 3) was significant higher in feeding group L3 ($P < 0.05$). An increased intake of CP and energy for group L3 was achieved, due to the feeding of lactulose. Male calves showed a similar average daily MR intake between treatment groups, though the female calves of group L1 ($P < 0.05$) showed a lower average daily MR intake ($P < 0.05$). Growth performance is presented in detail in Table 4. A positive trend on growth

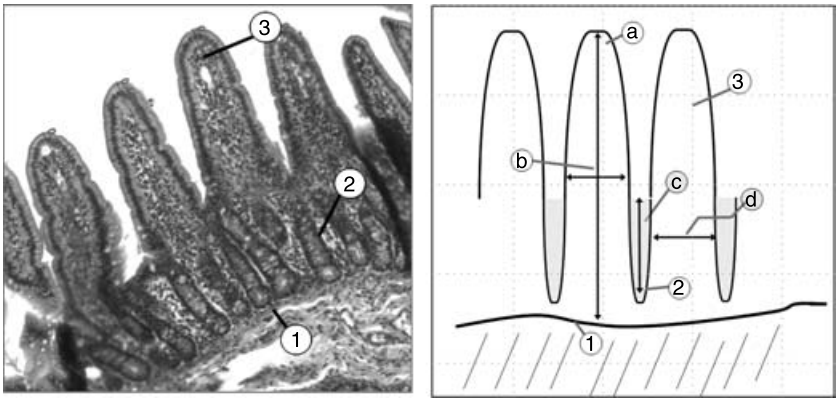

Figure 1 Morphological measurements in the intestine: (1) lamina muscularis mucosae; (2) crypt of Lieberkuhn; (3) villus. Measurements in the small and large intestine were combined pictured in the diagram. Small intestine: (a) villus height (from the tip of the villus to the lamina muscularis mucusae; (b) villus width (distance from villi-junction to the next – perpendicular to the height). Large intestine: (c) depth of crypt (from the tip to the lamina muscularis mucusae – in the large intestine villi is inexistent; (d) width of crypt (perpendicularly to the depth).

https://doi.org/10.1017/S1751731107661850 Published online by Cambridge University Press

Table 3 *Average daily food and nutritient intake*

Intake[†]	Experimental group			Pooled s.e.	Significance of group differences		
	L0	L1	L3		L0 v. L1	L0 v. L3	L1 v. L3
MR intake							
MR (g DM)	2080	2019	2199	45		*	**
male	1958	1934	2133	73			
female	2201	2104	2264	26	*		**
Energy (MJ ME)	35	34	37	1		*	**
Crude protein (g)	455	452	514	10		***	***
Ether extract (g)	410	398	433	9		*	**
Total intake							
Hay (g DM)	205	207	211				
Total food (kg DM)	2.3	2.2	2.4				
Energy (MJ ME)	37	36	39				
Crude protein (g)	477	475	545				
Crude fibre (g)	68	69	70				
Ether extract (g)	409	398	437				

[†] The milk replacer (MR) intake data show the mean values ± s.e. For the total intake, no s.e or significance values could be calculated because hay was offered to entire feeding groups (means are different between treatment groups as shown).

performance could be determined in group L3. Increasing dose of lactulose tended ($P < 0.1$) to increase average daily gain (ADG), especially for male calves. Feed efficiency was highly variable between the animals and not affected by the lactulose treatment.

Villus height and weight in the small intestine and crypts depths and widths in the large intestine

Villus height and width in the jejunum were unchanged in feeding groups (Table 5). However, in the ileum a reduction of villus height with increasing lactulose treatment ($P < 0.001$) was detected, with no change in villus width. In the control group, villus height and width between jejunum and ileum were not significantly different, but in both lactulose groups the villus height was significant lower in ileum than in jejunum (L1: $P < 0.01$; L3: $P < 0.001$). The villus width in the jejunum was decreased because of the 1% lactulose treatment ($P < 0.05$).

In both treatment groups L1 and L3 the caecal crypt depth was lower ($P < 0.001$) than in the control group. The lactulose treatment effect in L1 group was greater than in L3 ($P < 0.05$). In all groups the crypt depth was significant lower in the colon than in the caecum. There were no treatment effects on crypt width in the caecum, crypt depth and width in the colon and number of lymphatic follicle in the ileum (not shown).

Sex differences in intestinal morphology

In the ileum, the female calves in all feeding groups exhibited higher villus lengths than male calves, but only in group L3 was this difference significant ($P < 0.05$). Among all animals the caecum crypt depth was different between sexes ($P < 0.01$). In all groups the female calves showed lower crypt depths than the male calves. In the treatment groups (L1 and L3) the crypt depths of female calves were lower ($P < 0.001$). Among male animals only male calves of group L1 showed significantly lower crypt depth. The crypt width was lower in female calves of group L3 ($P < 0.05$). Results of the pairwise multiple comparison procedures are not shown.

Histomorphometry of follicles of Peyer's patches in ileum

The surface area of lymph follicles from Peyer's patches was decreased by lactulose treatment (Table 5). In the lactulose groups the ileum lymph follicles were smaller than in the control group (L1: $P < 0.05$; L3: $P < 0.01$). On closer examination, a significant difference between sexes was apparent ($P < 0.001$). The surface area of female calves in group L0 ($P < 0.05$) and L1 ($P < 0.001$) was larger than in male calves and lower in group L3 ($P < 0.01$). Within the female group the surface areas of lymph follicle in Peyer's patches were smaller in feeding group L3 than in group L0 and L1 whereas the surface area of male calves in feeding group L1 were smaller than in group L0 and L3 ($P < 0.01$).

Table 4 *Mortality and adjusted means ± s.e. of body-weight (BW) gain and feed efficiency of calves fed with milk replacer containing* E. faecium *(L0) or added with lactulose (L1 and L3) (means are not different ($P > 0.05$) between treatment groups)*

Variable[†]	Treatment groups[‡]			Pooled s.e.
	L0	L1	L3	
No of calves	14	14	14	0
Mortality	0	0	0	0
Initial BW (kg)	74	74	74	2
Final BW (kg)	244	245	255	6
ADG: weeks 1–19 (g/day)	1288	1276	1350	59
FE	0.59	0.61	0.59	0.07

[†] ADG = average daily gain; FE = feed efficiency, expressed as ADG (g/day)/milk replacer intake (g/day).
[‡] Only group difference L1 *v.* L3 approached significance for ADG ($P < 0.1$).

https://doi.org/10.1017/S1751731107661850 Published online by Cambridge University Press

Table 5 *Mean values for villus areas and heights and crypts depths in jejunum and ileum, size of lymph follicle area in Peyer's patches (ileum) and crypt depths in colon and caecum in calves fed milk replacer (MR) (L0), MR + 1% lactulose per MR (L1) and MR + 3% lactulose per MR (L3) (means are different between treatment groups as shown)*

	Experimental group				Significance of group differences		
Trait	L0	L1	L3	Pooled s.e.	L0 *v.* L1	L0 *v.* L3	L1 *v.* L3
Jejunum							
No.	42	36	41				
Villus width (μm)	148	142	148	5			
Villus height (μm)	920	884	979	26	‡	‡	‡
Villus height/width ratio†	6.3	6.2	6.9	0.3			
Ileum							
No.	38	42	42				
Villus width (μm)	157	160	156	5			
Villus height (μm)	896	770	727	24	***	***	
Villus height/width ratio†	5.7	4.9	4.7	0.3		*	
Peyer's patches (lymph follicle)							
No.	83	84	84				**
Area (μm^2)	363	318	303	14	*	**	***
Male	334	251	330	11	**		
Female	391	385	277	11		***	
Caecum							
No.	42	42	42				*
Crypts depth (μm)	586	515	542	10	***	***	
Crypts width (μm)	30	30	29	1.5			
Colon							
No.	42	42	41				
Crypts depth (μm)	507	478	496	12			
Crypts width (μm)	29	28	27	1.5			

† Values are means no. = 14 per group.
‡ Approaching significance ($P < 0.1$).

Discussion

Growth performance

Research on probiotics for cattle has increased in recent years and usually has shown a beneficial effect on the host. In the last 10 years, diverse effects, but not always statistically significant, have been found for feed intake, weight gain, decreased scouring, decreased faecal coliform count and reduced demand for antibiotic treatment (Fuller, 2005). Thus, in all experimental groups, we used a MR containing the probiotic bacteria *E. faecium* to achieve a possible improvement of health. The effect of lactulose as a prebiotic in animal nutrition was reported in studies with pigs (Kien *et al.*, 1999; Krueger *et al.*, 2002) and calves (Schroedl *et al.*, 2003; Landwirtschaftskammer Westfalen Lippe and Universität Leipzig, 2003). However, the effect of lactulose on pre-ruminant calves has not been investigated in detail.

In this study, ADG tended to be higher for L3 than L1 and was numerically higher for L3 than L0. Intake of MR was increased in group L3, so that average daily CP intake was about 13% higher for L3 than L0. This could represent a direct effect of lactulose on gut morphology or an indirect effect of 3% lactulose inclusion on sweetness. Quigley *et al.* (2006) reported that increasing the content of CP in MR increases ADG and efficiency of gain. Other trials in pigs observed no additional benefits from the use of lactulose (Krueger *et al.*, 2002). This is understandable since the initial status of the microbial colonisation of the intestine can differ widely between studies. Furthermore, the extent to which the well being and the performance are improved or maintained also depends on other factors, especially the composition of the diet, the sanitary conditions and the performance level (North Carolina Cooperative Extension, 2005). An accurate and reliable prediction of the lactulose efficacy in calves is not possible at this time.

GIT histology

As seen in previous studies, within the small intestine villus heights were the greatest in the jejunum (Blättler *et al.*, 2001), possibly due to enhanced differentiation from crypt cells to villus epithelial cells. This gut segment is thought to play a major role in absorption of the digestion products, because the intestinal surface is expected to be positively associated with absorptive capacity (Ganapathy *et al.*, 1994). Based on histomorphological analyses, our study indicates that the feeding of lactulose decreased the villus sizes only in ileum. These results disagree with those obtained by Pelicano *et al.* (2005), who found no differences in the ileal villus height with the use of prebiotics in broiler chickens. The continual reduction of the villus height in the ileum could be explained by a decreasing cell

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proliferation or/and an increasing apoptosis caused by the feeding of lactulose. Apoptosis is especially relevant in the GIT because it is an important process responsible for maintenance of the cellular balance between proliferation and death and crucial for normal morphology and function (Hall *et al.*, 1994). Prebiotics are proven to increase apoptosis in the intestine in order to exert a protective effect in carcinogenesis (Hughes and Rowland, 2001). Especially lactulose is said to reduce cell proliferation after supplementation for some days (Kien *et al.*, 1999). The synbiotic application of pro- and prebiotica could amplify this effect by reducing the number of aberrant crypts (Kien *et al.*, 2004). Although a higher lactulose concentration in the ileum than in the jejunum might be a reason for the effect on villus heights only in the ileum. In a study with pigs from Kamphues *et al.* (2003) and Branner *et al.* (2004) higher lactulose concentrations were measured at the end of the small intestine than in the jejunum. Lactulose concentration in chyme of calves deserves further study.

The decreasing crypt depth in the caecum due to lactulose-supplementation could also be explained by the already mentioned effect of prebiotics to decrease proliferative activity and to increase apoptotic rates. The production of short-chain fatty acids, like butyrate along with acetate and propionate, in the lumen of the hindgut by bacterial fermentation of lactulose was identified in previous work as a reason for this morphological effect (Mandal *et al.*, 2001). A number of different studies (and experimental paradigms) reported lower colonic cell proliferation by increased synthesis of butyric acid (Kien *et al.*, 1999; Klien *et al.*, 2006). In the large intestine this could possibly lead to a shortening of the crypts as was found in our study. In contrast to previous finding, Nilsson and Nyman (2005) and Fernandes *et al.* (2000) reported that lactulose yielded high proportions of acetic acid and low proportions of butyric acid. In further work analyses of the butyric acid concentration in the chyme should be conducted in calves.

Effect of lactulose on lymph follicles in Peyer's patches

A significant influence of lactulose-supplementation was shown on the gut-associated lymphoid tissue (GALT). In the GALT the Peyer's patches are the main component and especially present in the ileum part of the GIT (Norrman *et al.*, 2003). Ileal Peyer's patches are a primary lymphoid organ and play a major role in the development of B-cells (Norrman *et al.*, 2003). In both treatment groups a smaller size of the lymph follicles was observed with sex-specific differences (L1: $P < 0.05$; L3: $P < 0.01$) suggesting lower immunological activity throughout the lactulose rich feeding. The lymph follicles of male calves were only significantly affected in group L1. In contrast, only the female calves from group L3 with the highest lactulose feeding showed a significant dependence. The lymph follicle decreased significantly in the supplemented group ($P < 0.01$), which is explained by the stabilisation of the intestinal environment by the reduction of pathogen bacteria which leads to a reduced activation of the immune system. In this way the necessity of the host's immune system to react against harmful bacteria is decreased and this could lead to a reduced surface of lymphatic follicle in the intestine. Further analyses are necessary to confirm this assertion about the effect of lactulose on the Peyer's patches.

Conclusion

This study indicates that lactulose feeding in combination with *E. faecium* affects the morphology of the small and large intestine in pre-ruminant calves. GALT activation was reduced via the Peyer's patches in the ileum. Inclusion of 1% lactulose did not affect growth performance, but ADG tended to increase when lactulose inclusion was 3%. The effects of lactulose are obviously sex-specific: male calves tended to have higher body weight and female calves tended to have more changes in intestinal morphology in response to lactulose. Further studies are required to test the interaction between lactulose and probiotics such as *E. faecium*.

Acknowledgements

The research was supported by the H. Wilhelm Schaumann-Stiftung in Germany. Special thanks are due to the Landesuntersuchungsamt (LUA, Oberschleißheim) in Germany for their excellent paraffin embedding and many thanks to Milei GmbH providing the Lactusat, and Milkivit, Trouw Nutrition, located in Burgheim, Germany, for preparing the milk replacer.

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https://doi.org/10.1017/S1751731107661850 Published online by Cambridge University Press