Animal (2011), **5:7**, pp 1099–1106 © The Animal Consortium 2011
doi:10.1017/S1751731110002521

animal

Effects of the prebiotics inulin and lactulose on intestinal immunology and hematology of preruminant calves

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(Received 25 March 2010; Accepted 30 August 2010; First published online 27 January 2011)

Prebiotics are suggested as an alternative to antibiotics in animal rearing. Fermentable substances such as inulin or lactulose have been proposed to stimulate the immune system and health by modulation of the intestinal flora and its fermentation products. In this study, effects of inulin and lactulose on the intestinal health and hematology of calves have been investigated. Both prebiotics significantly decreased thrombocyte counts in peripheral blood. Only inulin was able to increase hemoglobin concentration and hematocrit. Total leukocyte count was decreased by lactulose while both prebiotics tended to lower monocyte proportions. mRNA expression of inflammation-related markers in the intestine was also affected by both prebiotics hinting at a decreased inflammatory status. This may be due to a possible decrease in intestinal pathogen load that remains to be verified. Only mRNA amounts of interleukin 8 were increased by lactulose in mesenteric lymph nodes. In the ileum, expression of a proliferation marker was increased by inulin while an apoptosis-related gene was increased by both prebiotics. The results of this study show a clear effect of prebiotics on certain parameters associated with animal health and performance that remain to be studied in detail in future investigations.

Keywords: calves, hematology, inulin, lactulose, prebiotic

Implications

Until now, research on prebiotic substances has been mainly carried out on laboratory animals such as rats. These studies mainly concentrated on intestinal bacterial communities, but in recent years some research on immunology and blood traits has also been carried out. This study now aims to give insights in effects of prebiotic substances on intestinal and systemic health in preruminant calves. To achieve this, hematology and expression of relevant genes in young cattle fed two different kinds of prebiotics have been investigated.

Introduction

Calf diseases, mainly diarrhea, are a severe problem during the early period of animal life and are often prevented or treated with antibiotics (Svensson *et al.*, 2003). However, antimicrobials in sub-therapeutic doses are also used to promote growth for meat production and this led to problems with resistant bacteria and antibiotic residues in meat (McEwen and Fedorka-Cray, 2002). Thus, antimicrobial growth promoters have been banned in the European Union (EG 183/2003). This caused adverse effects on animal health and thus increased the use of therapeutic antibiotics (Casewell *et al.*, 2003). Now, alternatives have to be found to stabilize gut health and to promote growth. These may include changes in nutrition, for example the inclusion of prebiotics (Lallès *et al.*, 2007). A prebiotic like inulin or lactulose has been defined as a non-digestible substance that beneficially affects the host by modulation of the intestinal flora (Gibson and Roberfroid, 1995).

Inulin is a natural β-(2-1)-linked fructo-oligosaccharide with up to 60 units common in plants used in the Western diet (Van Loo *et al.*, 1995). It has been shown to lead to a shift in the intestinal bacterial flora toward more beneficial bifidobacteria (Gibson *et al.*, 1995), to impede carcinogenesis (Femia *et al.*, 2002) or to stimulate the immune system (Schley and Field, 2002). In addition, it was shown to enhance growth performance of livestock (Van Loo, 2007).

Lactulose is a semi-synthetic disaccharide (Schumann, 2002) that is mainly fermented by beneficial bacteria like lactobacilli or bifidobacteria (Mitsuoka *et al.*, 1987). It has been commonly used to treat constipation (Attar *et al.*, 1999) or hepatic encephalopathy (Bircher *et al.*, 1966), and has been reported to have effects similar to inulin on carcinogenesis, immunology (Schumann, 2002) or animal growth performance (Fleige *et al.*, 2007a).

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This study investigated long-term treatment of calves with the prebiotics inulin and lactulose and their potentially beneficial effects on hematology and intestinal health. Inulin has been chosen since it is commonly accepted to have prebiotic characteristics, but only little is known about its effects on calves. Lactulose has been used in previous studies on calves in combination with the probiotic strain *Enterococcus faecium* (Fleige *et al*., 2007a, 2007b and 2009). In this study, its effects should be verified in the absence of additional effectors.

Material and methods

Animal husbandry and feeding

The animal housing and slaughtering procedures followed the actual German law on animal production and veterinary inspection (Bayerische Landesanstalt für Landwirtschaft (LfL), Grub, Germany).

Forty-two Holstein–Friesian bull calves were purchased from the Viehzentrum Waldkraiburg GmbH. Animals were transported to the experimental station Karolinenfeld (LfL, Institut für Tierernährung und Futterwirtschaft) and subdivided into three experimental groups ($n = 14$ per group). Weight (52.9 $\pm$ 6.2 kg) and age (22 $\pm$ 5 days) were balanced between groups.

The composition of diets is given in Table 1. Basic to all diets was the milk replacer Milkibeef Top (Milkivit, Trouw Nutrition, Burgheim, Germany). Control groups were fed the pure replacer, and the other groups were fed the same milk replacer iso-energetically and iso-nitrogenically enriched with 2% (dry matter) of either inulin (Beneo ST, Orafti, Tienen, Belgium) or lactulose (Lactusat, Milei GmbH, Leutkirch, Germany). This concentration is approximately equivalent to suggestions on the consumption of dietary fiber in human diets (Van Loo *et al*., 1995). In a previous study, lactulose has been used in similar concentrations (Fleige *et al*., 2009). Individual feeding was achieved by transponder automatic feeders (Förster Technik, Engen, Germany). During the experimental period of 20 weeks, the milk replacer concentration was rising from 125 to 200 g/l with daily intake volumes rising from 6 to 16 l. Calves had free group access to fresh drinking water and up to 300 g hay per day and animal. Since the calves were housed on straw, a further uptake of roughage could not be excluded. The animals were slaughtered after 20 weeks.

Tissue and blood sampling

Ten weeks after the beginning of the feeding experiment, and during the slaughtering process after 20 weeks of experimental diet, blood samples were taken from the jugular vein by venipuncture or during bleeding for examination by a veterinary laboratory (Vetmed Labor, Unterhaching, Germany). Venipuncture was included into the daily routine so that animals had free access to the milk replacer before sampling. Owing to transportation and a waiting period before slaughtering, animals were fasted during the second blood sampling. From each of the blood samples, 10 ml were also transferred to paxGene Blood RNA tubes (BD, Heidelberg, Germany) for RNA extraction. Tubes were stored at −20°C until extraction. Immediately after slaughter, organs were removed from the carcass. Small pieces were cut from central parts of the jejunum, ileum and colon and from the tip of the spleen. In addition, mesenteric lymph nodes were sampled. Intestinal samples were washed twice in physiological salt solution to remove digesta. All tissues were immediately flash frozen in liquid nitrogen. Samples were taken in duplicate and stored at −80°C until extraction to prevent RNA degradation.

RNA extraction and quality control

RNA extraction of whole blood stabilized in paxGene blood RNA tubes was performed using the paxGene blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA extraction of tissue samples was performed

Table 1 *Ingredients and analysis of nutrient and energy content of the diets (energy content of the milk replacer was estimated with the program Zifo; LfL, 2005)*

	Control	Inulin	Lactulose	Hay	Straw
50% Fat concentrate (%; 50% whey powder, 50% coconut/palm oil)	38.3	38.3	38.5		
Skimmed milk (%)	50.2	50.2	50.2		
Pregelatinized wheat starch (%)	4.4	2.8	3.5		
Whey protein concentrate (%)	4.1	3.5	–		
Beneo® ST (%)	–	2.2	–		
Lactusat (%)	–	–	4.8		
Vitamins, minerals, amino acid mix (%)	2.0	2.0	2.0		
80% soy bean oil/20% emulsifier (%)	0.985	0.985	0.985		
Aroma (%)	0.015	0.015	0.015		
DM (g/kg)	963	959	960	880	892
Crude ash (g/kg DM)	73	73	70	39	32
Crude protein (g/kg DM)	225	229	230	112	35
Ether extracts (g/kg DM)	210	208	211	15	13
Crude fiber (g/kg DM)	6	2	4	323	460
Energy (MJ/kg DM)	16.9	16.9	17.0	9.46	6.9

DM = dry matter.

Table 2 *Primer pairs, product sizes, gene identities and appropriate profiles for qRT-PCR. For every tissue one pair of reference genes was chosen to normalize crossing point values of target genes. All genes were measured in each tissue*

Target	Sequence	Size (bp)	Accession	Profile
		Reference genes		
ACTB				
For	AACTCCATCATGAAGTGTGACG	234	NM_173979	60/68
Rev	GATCCACATCTGCTGGAAGG			
GAPDH				
For	GTCTTCACTACCATGGAGAAGG	197	NM_001034034	60/68
Rev	TCATGGATGACCTTGGCCAG			
UBIQ				
For	AGATCCAGGATAAGGAAGGCAT	198	NM_174133	60/68
Rev	GCTCCACCTCCAGGGTGAT			
VPS4A				
For	CAAAGCCAAGGAGAGCATTC	222	NM_001046615	61/68
Rev	ATGTTGGGCTTCTCCATCAC			
GAK				
For	TCTGGGAAGTGGCAGAGAGT	294	NM_001046084	61/68
Rev	CGGCACGTCTGGTAGAAGAT			
RAB21				
For	CGGAAAATGTTGGGAAACG	229	XM_001249323	61/68
Rev	CATTGCCTTTTGCCCTCTC			
PMPCA				
For	CATCCCAGAATAAGTTTGGACAG	236	NM_001076964	61/68
Rev	AGAATCAGCAGACACAGCATACA			
		Genes of interest		
IL1B				
For	TTCTCTCCAGCCAACCTTCATT	198	NM_174093	61/68/80
Rev	ATCTGCAGCTGGATGTTTCCAT			
TNF				
For	CCACGTTGTAGCCGACATC	155	NM_173966	61/68/80
Rev	ACCACCAGCTGGTTGTCTTC			
IL8				
For	ATGACTTCCAAGCTGGCTGTTG	149	NM_173925	61/68/80
Rev	TTGATAAATTTGGGGTGGAAAG			
TGFB1				
For	ACGTCACTGGAGTTGTGCGG	166	NM_001166068	61/68/80
Rev	TTCATGCCGTGAATGGTGGCG			
IL10				
For	CCTGGAAGAGGTGATGCCAC	118	NM_174088.1	61/68/80
Rev	GTTTTCGCAGGGCAGAAAGCG			
IFN-g				
For	CTTGAATGGCAGCTCTGAGAAAC	173	NM_174086	61/68/80
Rev	GGCCTCGAAAGAGATTCTGAC			
FCAR				
For	GACAAACCCTTTCTCTCCACC	180	NM_001012685	61/68/80
Rev	ACAGGACCCAGAGTGAAGTC			
IL2RA				
For	ATGGAGCCAAGCTTGCTGATGT	171	NM_174358	61/68
Rev	TCTGCGGAAGCCTGTCTTGCA			
CD69				
For	GTCATTGATTCTAAAGAGGACATGA	137	NM_174014	60/68
Rev	AGGTTGAACCAGTTGTTAAATTCT			
CD4				
For	GATCGAGGTCTTGCCTTCAG	237	Multi (consensus)	61/68/80
Rev	GATCTGAGACATCCGTTCTGC			
CD8b				
For	ACTGTGTATGGCAAGGAGGTG	127	NM_001105344	61/68/80
Rev	GGGTATCCCAATGATCATGCAG			

Table 2 *Continued*

Target	Sequence	Size (bp)	Accession	Profile
PECAM1				
For	AAGGGAGGCATGACTGTGTC	187	NM_174571	61/68/80
Rev	TAATCACCTCGGACCTGGAG			
EGFR				
For	AACTGTGAGGTGGTCCTTGG	173	XM_592211	61/68/80
Rev	AAAGCACATTTCCTCGGATG			
BCL2L1				
For	GGCATTCAGCGACCTGAC	203	NM_001077486	61/68/80
Rev	CCATCCAAGTTGCGATCC			
BAX				
For	TCTGACGGCAACTTCAACTG	203	NM_173894	61/68/80
Rev	GGTGTCCCAAAGTAGGAGAGG			
CASP3				
For	GCAACGTTTCTAAAGAAGACCATAG	64	NM_001077840	60/68
Rev	CCATGGCTTAGAAGCACACAAATAA			
MKI67				
For	TGGCGAAGATGTGTTTCCT	130	XM_590872	60/68
Rev	CGTGCTCCTTGGTGTTTTC			

with TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA was extracted from small pieces (~50 μg) of tissue. Samples were not allowed to thaw until pieces were immersed in TriFast to avoid RNA degradation.

RNA quantity and purity of all samples (260 nm/280 nm absorption ratio) were measured with a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany). RNA quality was assessed using the Bioanalyzer 2100 with RNA Nano Chips (Agilent Technologies, Palo Alto, CA, USA).

Real-time quantitative reverse transcription PCR (qRT-PCR)
qRT-PCR was performed using a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) and the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). Specific primer sets were either adapted from Fleige *et al.* (2007b and 2009) or designed using primer3 *v.* 0.4.0 (http://frodo.wi.mit.edu/) and purchased from Eurofins MWG Operon (Ebersberg, Germany). The one-step qRT-PCR profile consisted of a reverse transcription (10 min at 50°C), a denaturation step (5 min at 95°C) and 40 cycles of amplification and quantification (with 15 s at 95°C, 30 s annealing at appropriate temperature, 20 s elongation at 68°C and a 15 s measurement step at 80°C). Primer sequences and PCR profile information are given in Table 2.

Data evaluation
Data of hematology examination are presented as mean ± s.e.m. with $n = 14$.

For qRT-PCR, the crossing point values were obtained with Rotor-Gene 6000 software version 1.7 (Corbett Life Science). For relative quantification of mRNA expression, a number of potential reference genes were measured and for each tissue the optimal pairs of reference genes were chosen using GenEx Pro Ver 4.3.4 (MultiD Analyses AB, Gothenburg, Sweden). The arithmetic mean of their crossing point values was used to normalize target gene values. The calculation of relative expression values was carried out using the 2-ΔΔCT method (Livak and Schmittgen, 2001). For each group, results are given in geometric means of expression ratios relative to the control group and r.e.m. Statistical analysis of gene expression data was carried out using the one-way ANOVA method of SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). In case of significant differences, a *post-hoc* Tukey–Kramer test was performed to further investigate group differences.

Results

Hematology
The results are listed in Table 3. Ten weeks after the start of the feeding regimes, animals of the lactulose group showed significantly reduced concentrations of peripheral blood leukocytes ($P = 0.005$) compared with the inulin and control group. Thrombocytes were significantly decreased by both experimental feedings compared with the control ($P = 0.008$). For the proportion of monocytes of total leukocytes, a trend for a decrease in both prebiotic groups could be found ($P = 0.073$).

At slaughter, all these effects were no longer visible, but instead both hemoglobin concentration ($P = 0.003$) and hematocrit ($P = 0.035$) were significantly increased in inulin-treated animals compared with control and lactulose-treated animals. At 10 weeks, a similar pattern was visible, which could not be statistically verified ($P = 0.286$ or $P = 0.158$, respectively).

RNA quality
RNA purity was regarded as good with an average A260/A280 ratio of 2.0 ± 0.01 for all tissues. Values >1.80 are considered as an indicator for high purity. RNA quality for all tissues was investigated with a Bioanalyzer 2100 (Agilent Technologies) and yielded an average RNA integrity number

Table 3 *Results of hematological examination of blood samples taken 10 weeks after beginning or during slaughtering*

	Control	Inulin	Lactulose	*P*-value
After 10 weeks				
Erythrocytes (10^6/μl)	8.8 ± 0.34	9.2 ± 0.22	8.9 ± 0.28	ns
Thrombocytes (10^3/μl)	0.75 ± 0.046[a]	0.55 ± 0.033[b]	0.66 ± 0.062[b]	0.008
Leukocytes (10^3/μl)	8.9 ± 0.53[a]	9.6 ± 0.84[a]	6.5 ± 0.51[b]	0.005
Granulocytes (%)[1]				
Basophil	0.9 ± 0.10	0.6 ± 0.14	0.6 ± 0.13	ns
Eosinophil	2.4 ± 0.52	2.1 ± 0.45	2.4 ± 0.53	ns
Segmented	28.2 ± 2.18	31.3 ± 2.57	31.6 ± 2.55	ns
Lymphocytes (%)[1]	61.4 ± 2.27	61.4 ± 3.25	60.1 ± 3.01	ns
Monocytes (%)[1]	6.4 ± 0.66	4.1 ± 0.75	4.8 ± 0.76	0.073
Hemoglobin (g/dl)	7.1 ± 0.38	7.5 ± 0.29	6.8 ± 0.33	ns
Hematocrit (%)	17.1 ± 0.82	18.6 ± 0.85	16.4 ± 0.76	ns
After 20 weeks				
Erythrocytes (10^6/μl)	11.2 ± 0.41	12.2 ± 0.31	11.8 ± 0.29	ns
Thrombocytes (10^3/μl)	0.46 ± 0.103	0.58 ± 0.074	0.65 ± 0.074	ns
Leukocytes (10^3/μl)	5.3 ± 0.51	6.2 ± 0.56	6.9 ± 0.85	ns
Granulocytes (%)[1]				
Basophil	0.6 ± 0.15	0.6 ± 0.14	0.8 ± 0.17	ns
Eosinophil	3.5 ± 0.81	3.8 ± 0.56	3.5 ± 0.85	ns
Segmented	41.2 ± 4.40	42.9 ± 3.89	47.6 ± 2.91	ns
Lymphocytes (%)[1]	49.7 ± 4.90	47.9 ± 3.64	42.6 ± 3.51	ns
Monocytes (%)[1]	4.6 ± 0.74	4.5 ± 0.53	4.5 ± 0.60	ns
Hemoglobin (g/dl)	7.7 ± 0.36[a]	8.7 ± 0.27[b]	8.1 ± 0.54[a]	0.003
Hematocrit (%)	21.8 ± 0.85[a]	24.2 ± 0.72[b]	21.6 ± 0.76[a]	0.035

[1]Of total leukocytes.
All values are presented as mean ± s.e.m. ($n = 14$). *P*-values are results of ANOVA analysis. Within rows, values without a common superscript additionally have been found significantly different by *post-hoc* Tukey–Kramer test ($P < 0.05$).

(RIN) of 6.9 ± 0.13. RIN is a measure for RNA quality ranging from totally degraded RNA (RIN = 1) to intact RNA (RIN = 10) (Fleige and Pfaffl, 2006; Bustin *et al*., 2009). A RIN of 5 was regarded as a minimal requirement for qRT-PCR. Single samples not sufficient for qRT-PCR were re-extracted, and when values did not reach adequate levels were omitted from analysis.

Reference gene pairs

Reference genes are regarded as acceptable if their mRNA expression is stable between treatment groups. GenEx Pro software (MultiD Analyses AB) is one possibility to choose ideal reference gene pairs to normalize mRNA expression data. Here, for each tissue, the best reference gene pair was chosen from all genes measured (Bustin *et al*., 2009). Thus, normalization was carried out with *actin β (ACTB)* and *G protein-associated kinase (GAK)* in the jejunum, vacuolar protein sorting 4 homolog A and *GAK* in the ileum, *ubiquitin (UBIQ)* and *glyceraldehyde-3-phosphate dehydrogenase* in the colon, *Ras-related protein Rab-21* and *peptidase (mitochondrial processing) alpha* in the spleen and *ACTB* and *UBIQ* in blood.

mRNA expression changes of inflammation and immune modulating factors

An overview of all significant gene expression changes found in this study is given in Table 4. Genes or tissues without significant changes are not shown.

In the jejunum, relative expression of the pro-inflammatory *tumor necrosis factor-α (TNF)* was influenced by the feeding group ($P = 0.018$). Lactulose induced a downregulation of its expression compared with the control as well as the inulin group (Tukey–Kramer test: $P = 0.025$ or $P = 0.017$, respectively).

In the ileum, the relative mRNA amount of anti-inflammatory *interleukin 10 (IL10)* was significantly influenced by the diet ($P = 0.048$). Specifically, it was upregulated by inulin and downregulated by lactulose, both relative to the control. *Post-hoc* test showed that expression levels were significantly different between the treatment groups ($P = 0.017$) but not between each of these groups and the control.

In the colon, the expression of *platelet endothelial cell adhesion molecule-1 (PECAM1)* mRNA was significantly increased by both dietary treatments ($P = 0.023$). The Tukey–Kramer test revealed significant differences in its expression between control and lactulose ($P = 0.017$) as well as between control and inulin ($P = 0.016$). Simultaneously, expression of the lymphocyte activation marker *interleukin 2 receptor alpha chain (IL2RA)* tended to decrease in lactulose-treated animals ($P = 0.058$).

In mesenteric lymph nodes, expression of the chemoattractant *interleukin 8 (IL8)* was significantly upregulated by lactulose ($P = 0.029$). The upregulation was significant when compared with control ($P = 0.027$) as well as to inulin ($P = 0.016$).

Table 4 *Changes in relative mRNA expression levels found in the* in vivo *study*

Tissue	Gene	Control	Inulin	Lactulose	*P*-value
Jejunum	*TNF*	1.0 ± 0.11^{ab}	1.1 ± 0.11^{b}	0.7 ± 0.10^{a}	0.018
Ileum	*IL10*	1.0 ± 0.19^{ab}	1.5 ± 0.17^{b}	0.8 ± 0.20^{a}	0.048
Ileum	*BAX*	1.0 ± 0.10	1.3 ± 0.10	1.3 ± 0.10	0.096
Ileum	*MKI67*	1.0 ± 0.27^{ab}	2.0 ± 0.35^{b}	0.8 ± 0.15^{a}	0.009
Colon	*PECAM1*	1.0 ± 0.06^{a}	1.3 ± 0.11^{b}	1.3 ± 0.14^{b}	0.023
Colon	*IL2RA*	1.0 ± 0.20	0.9 ± 0.32	0.7 ± 0.15	0.058
Mesenteric lymph node	*IL8*	1.0 ± 0.17^{a}	1.0 ± 0.10^{a}	1.4 ± 0.15^{b}	0.029

All values are presented as geometric mean $\pm$ s.e.m. ($n = 14$) of relative expression values with respect to the control levels. *P*-values are results of ANOVA analysis. Within a row means without a common superscript have been found significantly different after *post-hoc* Tukey–Kramer test ($P < 0.05$).

Interleukin 1β, transforming growth factor-β1, interferon-γ (IFN-g), receptor for Fc fragment of IgA, CD69, CD4 and *CD8b* were not influenced by the diet in any tissue analyzed. In the spleen and blood, no regulation of investigated genes were found (data not shown).

mRNA expression changes of proliferation and apoptosis-related genes

Changes in expression of proliferation or apoptosis-related genes were found only in the ileum (Table 4). There, the proliferation marker *MKI67* was significantly influenced by both prebiotics ($P = 0.009$). Inulin led to an upregulation of its expression relative to the control ($P = 0.028$), whereas lactulose decreased it slightly. Such expression was significantly different between the inulin and the lactulose group ($P = 0.003$) but not between both and the control. Simultaneously, lactulose and inulin showed a weak tendency to increase the relative expression of pro-apoptotic *BCL2-associated X protein (BAX*; $P = 0.096$).

BCL2-like-1, epidermal growth factor receptor and *Caspase 3* were not influenced by the diet (data not shown).

Discussion

Until now, only a few studies on prebiotics have been completed with calves, and to the authors' knowledge, only one other long-time study was performed where changes in gene expression and hematological traits during feeding of lactulose have been investigated. There, the authors described a decrease in thrombocyte numbers in 3% lactulose-fed calves (Fleige *et al*., 2009) that could be supported for both prebiotics in this study after 10 weeks of feeding. A high rate of thrombocyte aggregation is considered as one of many risk factors for coronary heart disease and has been shown to be negatively correlated with intakes of higher amounts of dietary fiber in humans (Pietinen *et al*., 1996) and rats (Bagger *et al*., 1996).

At the end of the experimental period, only hemoglobin concentrations and hematocrit were affected by inulin feeding. Ohta *et al*. (1998) reported that these two parameters were significantly reduced after gastrectomy and subsequent malabsorption of iron in rats, but the levels could be recovered by 0.75% oligofructose feeding. Iron deficiency is a common problem in veal calves fed only milk replacer and no solid feed or fiber-rich supplementation (Cozzi *et al*., 2002). Inulin feeding could have had improving effects on iron absorption capabilities as was shown previously in rats (Ohta *et al*., 1995).

Immune- and inflammation-modulating effects of prebiotic substances – especially for inulin and oligofructose – have been reported repeatedly (e.g. Field *et al*., 1999; Schley and Field, 2002; Hosono *et al*., 2003). In this study, leukocyte counts in peripheral blood were decreased in lactulose-treated calves and monocyte numbers were decreased in both treatment groups. A similar effect has been found when mannanoligosaccharides were fed to dogs (Middelbos *et al*., 2007). In addition, the expression of *IL2RA* – an activation marker on the surface of lymphocytes – tended to be lowered in the colon of lactulose-treated calves compared with the control. Fleige *et al*. (2009) found a similar lowering of its expression in the mesenteric lymph nodes of lactulose-treated calves. Middelbos *et al*. (2007) suggested that a lowering of the infectious load in the intestine due to the feeding of mannan moieties may decrease the necessity of activated immune cells in the vicinity. This may also be reflected in peripheral blood. A similar decrease in pathogen load in the intestine is also attributed to prebiotics such as inulin or lactulose (Bovee-Oudenhoven *et al*., 1997; Flickinger *et al*., 2003).

A similar pattern as for leukocyte counts and *IL2RA* expression could be found for *TNF* expression in the jejunum. Lactulose significantly reduced its expression confirming again a decreased activation of an inflammatory or immune response in the intestine. A similar effect on *TNF* has been found before in a rat colitis model after treatment with lactulose (Camuesco *et al*., 2005). In contrast, pro-inflammatory *IL8* was significantly enhanced in the mesenteric lymph nodes of animals in the lactulose group. Fleige *et al*. (2009) also reported increases of *IL8* expression in the spleen and mesenteric lymph nodes of calves after feeding of lactulose, but these results were not statistically significant. *IL8* expression can be increased by lipoteichoic acid of gram-positive pathogens (Standiford *et al*., 1994) that may have been transported to the mesenteric lymph nodes by antigen-presenting cells. But since no induction of *IL8* was found in gut tissues, where these bacteria should show a first effect, it has to be considered whether other factors possibly

independent of gut flora may be responsible for the significant increase in *IL8* expression in the mesenteric lymph nodes of lactulose-fed calves.

Anti-inflammatory *IL10* showed an increased expression in the ileum of inulin-treated calves, especially when compared with lactulose-treated animals. The ileum wall is interstratified with Peyer's patches, parts of the gut-associated lymphoid tissue. Cells from these patches have been shown to release anti-inflammatory cytokines such as *IL10* after stimulation with probiotics, prebiotics or fermentation products thereof (Säemann *et al.*, 2000; Hosono *et al.*, 2003; Roller *et al.*, 2004). Therefore, it seems probable that fermentation products deriving from inulin degradation or secretory molecules from bacteria stimulated by inulin feeding are causative agents in this increase of *IL10* expression.

PECAM1 is a cell-surface molecule of leukocytes and endothelial cells and is crucial for the transmigration of leukocytes through the capillary wall into inflamed tissue (Wakelin *et al.*, 1996), which rather stands for pro-inflammatory action. Nevertheless, it has been found to be downregulated on endothelial cells in typical pro-inflammatory situations such as stimulation with *TNF* or *IFN-g* without effect on leukocyte transmigration (Shaw *et al.*, 2001). In addition, this molecule also plays a role in angiogenesis (DeLisser *et al.*, 1997), and therefore an upregulation could also mean an increase in perfusion of the surrounding tissue. A stimulation of angiogenesis in gastric ulcer healing by probiotic bacteria – targets of prebiotics – has been found previously (Lam *et al.*, 2007) and may be related to the present finding.

Expression of the proliferation marker *MKI67* in the ileum was increased in the inulin group. Interestingly, morphological investigations during the same study rather showed a slight decrease in the villus length and number of proliferative cells in the same group (Masanetz *et al.*, 2010). Maybe the main *MKI67* expression was not located in the epithelial layer, which was investigated by microscopy, but in other parts of the gut wall. Nevertheless, both enhancement (Brunsgaard and Eggum, 1995) and decline (Femia *et al.*, 2002) of gut epithelial proliferation have been described after treatment with different prebiotic substances in animal models. Simultaneously, a trend for an increased expression of *BAX* mRNA has been found in the ileum in both prebiotic groups. It has been reported before that butyrate – a fermentation product of prebiotic substances – is able to increase apoptosis via upregulation of *BAX* expression (Mandal *et al.*, 2001). This is fitting to the proposed anti-carcinogenic effects described for inulin (Femia *et al.*, 2002).

Differences between effects of both prebiotic substances or different gut segments may be due to fermentation properties of the substances themselves (Branner *et al.*, 2004). In addition, the density and composition of calf intestinal flora also vary greatly within the gut with decreasing anaerobic bacteria counts and a shift toward bifidobacteria from the rumen to the colon (Collado and Sanz, 2007). In addition, diverse stimulatory effects on beneficial bacterial subpopulations and fermentation product profiles were reported for inulin and lactulose (Rycroft *et al.*, 2001).

Changes in hematological traits between the samplings at 10 and 20 weeks may be correlated to age-related changes that have been demonstrated previously (Mohri *et al.*, 2007). For example, similar to this study, a significantly higher platelet count was found in young calves that decreased to adult control levels over time (Brun-Hansen *et al.*, 2006). Differences in regulations of blood characteristics may also play a tremendous role in the effectivity of prebiotic substances. In future studies, changes of hematology in growing cattle fed different diets should be investigated in detail.

In conclusion, both prebiotic substances used in this study showed effects on immune regulation and inflammation both systemically and locally in the gut of calves. Inulin and lactulose decreased signs of immune activation and increased anti- or lowered pro-inflammatory signals, respectively. Presumably, these effects were generated by a decline in pathogen load in the intestine commonly attributed to prebiotic treatment. Effects of both substances on proliferation and apoptosis are rather diverse and remain to be studied in detail. In addition, positive effects on hemoglobin and hematocrit have only been shown for inulin. These presumed effects on iron absorption are highly interesting and remain to be studied in future experiments, especially with a look at other mineral absorption capabilities. On the whole, a beneficial effect of prebiotic feeding on veal calf performance may be expected for both inulin or lactulose regarding the results of this study.

Acknowledgments

The current research work was supported by grants from the 'Vereinigung zur Förderung der Milchwissenschaftlichen Forschung' (Germany). Special thanks go to Milei GmbH for providing the lactulose preparation, to Milkivit, Trouw Nutrition (Burgheim) for the preparation of the milk replacer and to Orafti for the Beneo® ST.

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