

Lactulose: effect on apoptotic- and immunological-markers in the gastro-intestinal tract of pre-ruminant calves

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ABSTRACT: The study was conducted to elucidate the effects of orally administered lactulose in combination with *Enterococcus faecium* on immune response of the intestinal tract in pre-ruminant calves. The mRNA expression of pro- and anti-inflammatory cytokines and proliferation and apoptosis markers were investigated in jejunum, ileum, colon and caecum. Simmental calves were fed diets containing 1% (L1) or 3% (L3) lactulose and the probiotic strain of the genus *E. faecium*, and compared with a non treated control group. Primarily the high dose feeding with lactulose showed an effect on several mRNA gene expression parameters. In the jejunum a down-regulation of the anti-apoptotic marker Bcl-xl was determined and IL-10 mRNA gene expression was 2.6-fold up-regulated ($P < 0.05$). In the colon a 1.9-fold ($P < 0.05$) up-regulation of IL-10 and only in caecum an about 2-fold increase of TGF- β_1 ($P < 0.05$) was found for both lactulose feedings. Caspase 3 was up-regulated in caecum only in the 3% lactulose treated group ($P < 0.05$). The enhanced apoptotic rate of caspase 3 seems to be associated with a decrease in crypt depth due to lactulose supplementation. The results indicated that mainly the high 3% lactulose dose in probiotic-fed calves has an affect on the intestinal immune function and on diverse apoptotic markers.

Keywords: intestine; morphology; health

Nutritional and disease problems in calves continue to be an important part of dairy practice and are an important and increasing source of revenue for beef practitioners. In recent years there have been many advances in the prevention and treatment of calf problems. A large number of feed products are available to prevent scours and promote gut health and animal growth rates. The actual benefits of these products are hard to quantify, but clearly they modify and protect the gut health in periods of stress and disease. The most common milk additives are probiotics, prebiotics, rennet, sodium bentonite, antibiotics, vitamins and minerals (Schouten, 2005).

Prebiotics like lactulose containing fructose have been used in the diets of calves and pigs to improve intestinal health and to reduce the incidence of diseases (Flickinger et al., 2003; Patterson and

Burkholder, 2003). The prebiotic lactulose is a synthetic disaccharide which is neither absorbed nor suggested to be metabolized in the upper gastro-intestinal tract (GIT) (Macfarlane et al., 2006). More recently, prebiotics have been proposed as a mean to manipulate the bacterial flora of the intestinal tract of animals to potentially reduce the incidence of diseases (Bohmer et al., 2005), and they have direct effects on immune responses (Macfarlane and Cummings, 1999; Pie et al., 2007). Physiological effects of not absorbable carbohydrates include increased fecal bulk, increased short chain fatty acids (SCFA) production, and modification of bacterial populations (Jenkins et al., 1999; Gibson et al., 2005; Tuohy et al., 2005). SCFA produced by intestinal bacteria and the colonic microbes affect mucosal and systemic immunity in the host (Hooper et al., 2002). Bacterial products with immunomodulato-

ry properties include also endotoxic lipopolysaccharides, peptidoglycans, and lipoteichoic acids (Parvez et al., 2006). Whether prebiotics modulate the immune response directly, by affecting the composition of the intestinal flora and thus affecting the gastro-intestinal tract (Wilson et al., 1996), or indirectly by the fermentation to short chain fatty acids (SCFA), is presently unknown.

As well as modulating gut flora composition, prebiotics may exert cancer protective effects at the cellular level following SCFA formation. SCFA induce apoptosis in colon adenoma and cancer cell lines (Hague et al., 1994). In the colonic crypts, apoptosis maintains the balance in cell number between newly generated and surviving cells and at the luminal surface where differentiated epithelial cell are exfoliated (Potten, 1992). Previous investigations showed an effect of lactulose feeding on the morphology of the small and large intestine in pre-ruminant calves (Fleige et al., 2007). Based on histomorphological analyses, the study indicates that lactulose reduced the villus sizes in the ileum and decreased crypt depth in the caecum.

The aim of the present study was to determine the intestinal immune functions induced through a prebiotic treatment by lactulose in pre-ruminant calves. Furthermore, changes in the intestinal mRNA gene expression of apoptotic markers were analyzed to confirm previous morphological investigations (Fleige et al., 2007). Calve diseases have significant effect on the profitability of every calf raising enterprise. During weaning, dairy calves fed with milk replacer are susceptible to many pathogens that cause diseases. We hypothesized that lactulose in probiotic-fed calves would modify immune responses in the intestine and have an effect on the mRNA gene expression of pro- and anti-apoptotic markers. Therefore, we fed calves with milk replacer (MR) supplemented with *E. faecium* and different doses of lactulose. We assessed a broad spectrum of pro- and anti-inflammatory cytokines and apoptosis marker in the small and large intestine.

MATERIAL AND METHODS

Experimental animals and treatments

Forty-two Simmental calves from various farms were directly bought from the Simmental breeding organization (Zuchtverband für oberbayerisches Alpenfleckvieh e.V.) in Miesbach, Germany. All

calves were single-born and were separated from their dams immediately after birth. They were divided into three homogenous experimental groups with a balanced weight of 74.4 ± 2.1 kg, age ranged at 39 ± 2 days and gender was 50% male plus 50% female. During the feeding experiment, all calves were fed with MR from Milkibee Top (Milkivit, Trouw Nutrition, Burgheim, Germany) and further on 10^9 CFU (colony forming units) *Enterococcus faecium*. Standard feeding group (Control) was only fed with the MR, and served as control. The other two treatment groups were fed with MR enriched by 1% (L1) and 3% lactulose (L3). Therefore, the MR for group L1 and L3 was mixed with 2.5% and 7.5% Lactusat (Milei GmbH, Germany) which contains 42% lactulose. To guarantee a balanced feeding regime, in terms of energy and protein concentration, the Lactusat was added in exchange against whey powder. Calves of all feeding groups received MR in volumes up to 17.5 l/day in the experimental period of 19 weeks 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, with a continuous increase up to 250 g/l at the end of the study. All calves had free access to fresh water and 0.5 kg hay per day. After the dosing period of 133 ± 8 days the calves were slaughtered. The animal housing, sampling and euthanasia employed in this study followed the actual German law on animal production and veterinary inspection (LfL, Grub, Germany).

Tissue collection

Samples of the middle part of jejunum, ileum, colon and caecum were obtained at the time of slaughter. All animals were healthy and had no visible pathological signs in gastro-intestinal tract (GIT). Immediately after collection the tissues were placed into individually labeled cryotubes and frozen in liquid nitrogen. Cryotubes were removed from liquid nitrogen in the laboratory and stored at -80°C until analysis.

RNA extraction and RNA quality control

Total RNA from each tissue sample (~50 mg) was extracted by using TriFast reagent (Peqlab,

Erlangen, Germany) as recommended by the manufacturer. The quality and quantity of extracted total RNA were assessed using UV spectrophotometry. Integrity of the extracted total RNA was verified by optical density A260nm/A280nm absorption ratio. A second quality control was done by a micro-fluidic capillary electrophoresis (Fleige and Pfaffl, 2006). 100 ng of each experimental RNA sample was loaded onto a RNA 6000 Nano Chip and assayed using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). A commercially available and optimized RNA length standard ladder (Ambion, Austin, TX) during electrophoresis allows the evaluation of sizes of RNA bands. The algorithm assigns a RIN number score from 1 to 10, whereas level 10 represents a completely intact RNA, and 1 presents a highly degraded RNA. In this way, interpretation of an RNA integrity shown in detail as electropherogram was facilitated and comparison of samples were enabled.

Real-time qRT-PCR

One step real-time qRT-PCR were performed by using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA) by a standard protocol in a Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia). The master-mix was prepared (to the indicated end-concentration) as follows: 5 µl 2X SYBR Green Reaction Mix, 0.5 µl forward primer (10 pmol), 0.5 reverse primer (10 pmol) and 0.2 µl SYBR Green One-Step Enzyme Mix. Bovine sequence-specific primers were designed by using the HUSAR program (DKFZ, Heidelberg, Germany) and were synthesized by a commercial facility (MWG Biotech, Ebersberg, Germany). Sequences used for primer design were obtained from public databases (GenBank, National Center for Biotechnology Information) as coding DNA sequence (CDS). The primer sequences are listed

Table 1. Primers used for real-time qRT-PCR

Identity	Sequence (5' → 3')		Size (bp)	Accession No.
β-Actin	FOR	AAC TCC ATC ATG AAG TGT GAC G	202	AY141970
	REV	GAT CCA CAT CTG CTG GAA GG		
GAPDH	FOR	GTC TTC ACT ACC ATG GAG AAG G	197	U85042
	REV	TCA TGG ATG ACC TTG GCC AG		
U biquitin	FOR	AGATCCAGGATAAGGAAGGCAT	198	Z18245
	REV	GCTCCACCTCCAGGGTGAT		
IL-1β	FOR	TTC TCT CCA GCC AAC CTT CAT T	198	M37211
	REV	ATC TGC AGC TGG ATG TTT CCA T		
TNF-α	FOR	CCA CGT TGT AGC CGA CAT C	197	NM173966
	REV	CCC TGA AGA GGA CCT GTG AG		
IL-8	FOR	ATG ACT TCC AAG CTG GCT GTT G	149	AF232704
	REV	TTG ATA AAT TTG GGG TGG AAA G		
TGF-β ₁	FOR	ACG TCA CTG GAG TTG TGC GG	267	XM592497
	REV	TTC ATG CCG TGA ATG GTG GCG		
IL-10	FOR	CCT GGA AGA GGT GAT GCC AC	132	U00799
	REV	GTT TTC GCA GGG CAG AAA GCG		
EGFR	FOR	AAC TGT GAG GTG GTC CTT GG	173	AY486452
	REV	AAA GCA CAT TTC CTC GGA TG		
PECAM-1	FOR	AAG GGA GGC ATG ACT GTG TC	187	NM_174571
	REV	TAA TCA CCT CGA ACC TGG AG		
Bcl-xl	FOR	GGG ATT CAG CGA CCT GAC	203	AF245487
	REV	CCA TCC AAG TTG CGA TCC		
BAX	FOR	TCT GAC GGC AAC TTC AAC TG	194	L22473
	REV	AAG TAG GAG AGG AGG CCG TC		
Caspase 3	FOR	GCA ACG TTT CTA AAG AAG ACC ATA G	64	AY57500
	REV	CCA TGG CTT AGA AGC ACA CAA ATA A		

in Table 1. For one-step qRT-PCR 3.8 µl total RNA (10 ng/µl) was added as PCR template to 6.2 µl total volume of master-mix and for the reaction the following cycling protocol was used: (i) reverse transcription (10 min at 55°C); (ii) denaturation program (5 min at 95°C); (iii) 40 cycles of amplification and quantification (15 s at 95°C; annealing for 30 s at 60°C; elongation for 20 s at 68°C with a single fluorescence measurement); (iv) melting curve program (60–99°C with a heating rate of 0.5°C per second and a continuous fluorescence measurement); (v) cooling program down to 40°C.

Data evaluation

Crossing points (CP) and single run efficiency (E) were achieved for each analyzed sample and gene using the Rotor-Gene 3000 software version 6.0 (Corbett Life Science). The relative mRNA levels were calculated by using the single-run-specific efficiency-corrected relative expression model (Pfaffl, 2001). To assess the effect of the prebiotic treatment on cytokine gene expression in the different tissues the relative expression ratio (R) was calculated compared to the arithmetic mean expression of three reference genes (β -actin, GAPDH, Ubiquitin). To factor the PCR efficiency into the analyses each analyzed sample was calculated apart with the sample specific efficiency, according to the calculation model shown in Figure 1. Values were expressed as means \pm SEM. All statistical analyses were performed with Sigma Stat 3.0 (SPSS Inc. Chicago, IL, USA) using the one-way ANOVA.

RESULTS

RNA quality

The purity of the total RNA extracted was verified by an average A260/A280 ratio of 1.87 (range 1.76–1.98). An A260/A280 ratio greater than 1.8 is usually considered an acceptable indicator of high

quality RNA. The second quality control was done by the Bioanalyzer 2100. The average total RNA quality of all samples studied was a RIN of 7.4 ± 0.7 .

Gene expression changes of reference genes

For an accurate normalization of real-time qRT-PCR data stable and optimal reference genes are essential (Vandesompele et al., 2002). Ubiquitin, GAPDH and β -actin showed a constant expression level in all studied tissues and were determined as optimal reference genes.

Gene expression changes of pro- and anti-inflammatory cytokines

The high dose feeding (L3) induced mRNA expression changes for two analyzed anti-inflammatory cytokines in different parts of the intestine. IL-10 mRNA gene expression was 2.6-fold up-regulated ($P < 0.05$) in the jejunum and 1.9-fold up-regulated in the colon (Figure 2). A 2.1-fold increase of transforming growth factor β_1 (TGF- β_1) in the caecum was found among lactulose treatment ($P < 0.05$). Similarly the L1 group showed a 1.9-fold trend of up-regulation of TGF- β_1 in the caecum (Figure 2). No mRNA expression changes for further pro-inflammatory markers (IL-1 β , IL-8, TNF- α) were induced by lactulose feeding (data not shown in figures).

Gene expression changes of pro- and anti-apoptotic molecules

The oral application of lactulose induced only in the high dose treatment group (L3) mRNA expression changes for anti-apoptotic factors. A feeding with 3% lactulose affected a down-regulation about 76% of the Bcl-xl mRNA gene expression (Figure 3) in the jejunum ($P < 0.05$) and an up-regulation about 70% of caspase 3 ($P < 0.05$) in the caecum (Figure 3).

$$R = \frac{(E_{RG1})^{CP_{treated}} + (E_{RG2})^{CP_{treated}} + (E_{RG3})^{CP_{treated}}}{(E_{TG})^{CP_{treated}}} \div \frac{(E_{RG1})^{CP_{control}} + (E_{RG2})^{CP_{control}}}{(E_{TG})^{CP_{treated}}}$$

Figure 1. For normalization of each analyzed target gene (TG) in the presented study the arithmetic mean expression of three reference genes (β -actin, GAPDH, and ubiquitin) was used. Further the efficiency of mentioned genes was included in the calculation model

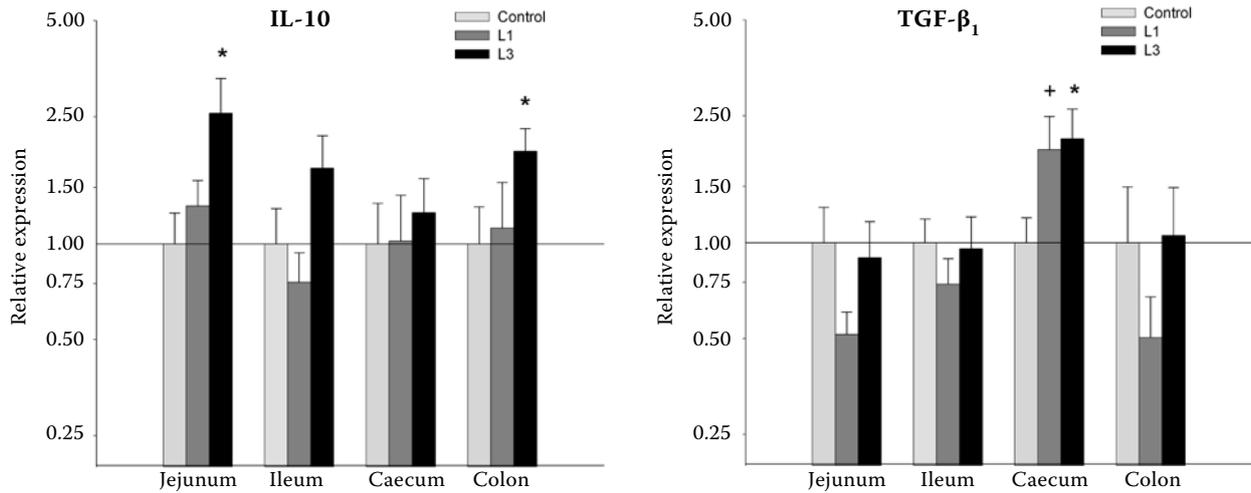


Figure 2. IL-10 and TGF-β₁ mRNA expression changes due to lactulose feeding. Data are presented as relative expression in means ± SEM (n = 14). Up-regulated genes are shown as values higher than 1 and down-regulated genes as values lower than 1. Significant effects of lactulose are marked with an asterisk (*P < 0.05) and trends of regulation are shown with plus (+0.05 < P < 0.1)

No mRNA expression changes for PECAM, EGFR and BAX were induced by lactulose feeding (data not shown in figures).

DISCUSSION

The objective of the present study was to investigate the effects of lactulose as a prebiotic in probiotic-fed calves on the intestinal immune functions. Furthermore, the effect on pro- and anti-apoptotic

factors in the intestine should be assayed to ensure previous histological investigations (Fleige et al., 2007). Animal studies, as well as data obtained from *in vitro* cell culture systems, have underlined the potential of certain prebiotics to protect against inflammatory and cancerous processes in the large intestine. The biochemical mechanisms are still unknown, but both the promotion of lactic acid-producing bacteria and the production of SCFA, particularly butyrate during the fermentation of prebiotics, could be key factors.

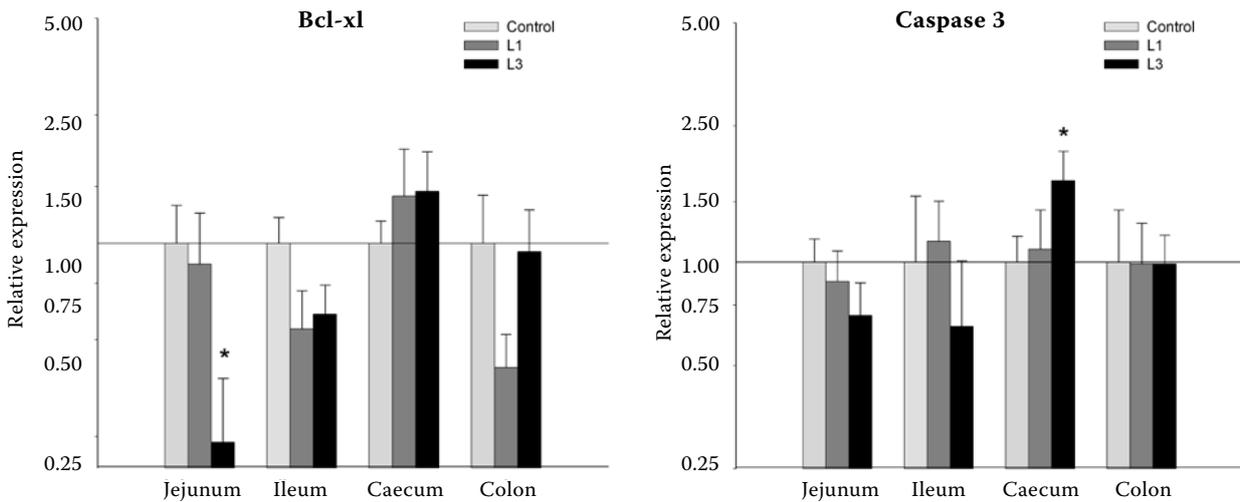


Figure 3. Effect of lactulose on the anti-apoptotic factor Bcl-xl and apoptotic factor caspase 3 in various intestinal calf tissues, compared to the untreated control group. Data are presented as relative expression in means ± SEM (n = 14). Up-regulated genes are shown as values higher than 1 and down-regulated genes as values lower than 1. Significant effects of lactulose are marked with an asterisk (*P < 0.05)

Stimulation of the GALT (gut associated lymphoid tissue) via prebiotics and probiotics might influence immune modulation because of their ability to enhance production of IL-10 and TGF- β_1 . Both are anti-inflammatory cytokines, which might have an essential role in dampening allergen-induced responses. Overall, the 3% lactulose feeding significantly stimulated IL-10 production in the jejunum and colon. IL-10 is primarily produced by T-helper2/T-regulatory lymphocytes and dendritic cells. It binds to a specific receptor on intestinal epithelial cells and regulates the contribution of epithelial cells to the inflammatory and immune response in the digestive tract (Denning et al., 2000). IL-10 can inhibit antigen specific proliferation and cytokine secretion by Th1 lymphocytes and has down regulatory effects on macrophages and dendritic cells, such as suppression of activation and IL-12 production (Moore et al., 1993; Tripp et al., 1993). IL-10 can also prevent interferon- γ induced disruption of colonic epithelial barriers (Madsen et al., 1997). Recent studies with mice also reported that consuming diverse prebiotics (fructooligosaccharide or inulin enriched with oligofructose) enhanced IL-10 production (Hosono et al., 2003; Roller et al., 2004). Furthermore, the high dose lactulose feeding significantly stimulated TGF- β_1 production in the caecum. TGF- β_1 is a multifunctional cytokine that regulates many diverse cellular processes including proliferation, apoptosis, differentiation (Tanigawa et al., 2005) and immune regulation. Several reports suggest that TGF- β_1 may function as a regulator of epithelial morphogenesis in the GIT. TGF- β_1 is an effective inhibitor of proliferation and tended to have its strongest inhibitory effects in the lower (stem cell) regions of the crypts (Potten et al. 1995), which could be a reason for the shortening of crypts in the caecum as was found in previous investigations by Fleige et al. (2007). Production of IL-10 and TGF- β_1 leads to activation of regulatory T cells, which in turn inhibit the immune response and induce mucosal tolerance (Maloy and Powrie, 2001; Singh et al., 2001). Generally, probiotics increase the production of intestinal anti-inflammatory cytokines (such as IL-10 and TGF- β_1), while reducing the production of pro-inflammatory cytokines (Ewaschuk and Dieleman, 2006) and prebiotics could amplify this effect. Whether prebiotics modulate the immune response directly or indirectly, by affecting the composition of the intestinal flora and thus affecting the gut associated lymphoid tissue, or by

producing SCFA, is presently unknown. Postulated are effects on luminal micro ecology, mucosal barrier function, and immunoregulation.

In the small intestine we have shown that oral administration of lactulose has an suppressive effect on the anti-apoptotic marker Bcl-xl in the jejunum ($P < 0.05$). Bcl-xl is the dominant regulator of apoptosis. It is known as the survival protein because the long form of Bcl-xl has cell death repressor activity (Sattler et al., 1997). The interaction with EGFR and its vital role in the apoptosis pathway makes Bcl-xl to an interesting candidate gene. Apoptosis is recognized as an important process responsible for maintenance of the cellular balance between proliferation and death. This form of cell death can be induced by a wide range of cellular signals, which lead to activation of cell death machinery within the cell and is characterized by distinct morphological changes (Aschoff et al., 2004). Apoptosis is especially relevant in the GIT, as the mammalian intestinal mucosa undergoes a process of continual cell turnover that is essential for maintenance of normal gut epithelial function. Dysregulated apoptosis is seen in a number of pathological conditions in the GIT (Ramachandran et al., 2000).

Therefore, a decreased anti-apoptotic rate seems to be associated with an increase in villus heights in the jejunum of lactulose treated calves. Fleige et al. (2007) reported about a trend of higher villus heights in the jejunum in 3% lactulose fed calves. Furthermore, the high lactulose feeding has up-regulated the apoptotic molecule caspase 3 in the caecum ($P < 0.05$). This result goes in line with previous histological investigation by Fleige et al. (2007), who found a decreasing crypt depth in the caecum due to lactulose-supplementation. The prebiotics are proven to increase apoptosis in the intestine (Hughes and Rowland, 2001). Especially lactulose is said to reduce cell proliferation after supplementation for some days (Kien et al., 1999). The production of SCFA 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).

CONCLUSION

This study indicates that a high dose lactulose feeding in combination with *E. faecium* affects the intestinal immune function. The higher mRNA expression of IL-10 and TGF- β_1 leads to

induce mucosal tolerance. Consequently, the calves might be more resistant to diseases. Further studies with calves are required to confirm these data. Morphological changes due to lactulose could be explained by the up-regulation of caspase 3 and TGF- β_1 in the large intestine.

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