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Lactulose in combination with *Enterococcus faecium*: Protective role in calves

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CONTENT

ABBREVIATIONS

ADG	average daily weight gain
ANOVA	analysis of variance
bp	base pairs
BW	body weight
CD	cluster of differentiation antigen
CFU	colony forming units
CP	crossing point
Ct	cycle threshold
E	efficiency
E. faecium	*Enterococcus faecium*
EDTA	ethylendiamintetraaceticacid
EGFR	epidermal growth factor receptor
EMBL	European Molecular Biology Laboratory
Fig.	figure
Fru	fructose
Gal	galactose
GAPDH	glycerinaldehyd-3-phosphat-dehydrogenase
GALT	gut-associated lymphatic tissue
GIT	gastrointestinal tract
Glu	glucose
IFN-γ	interferon gamma
IL	interleukin
IgA	immunoglobulin A
IgA FcR	IgA Fc receptor
L0	control group
L1	1% lactulose treated group
L3	3% lactulose treated group
mLN	mesenterial lymph nodes
mRNA	messenger RNA
MR	milk replacer
n	number
OS	oligsaccharide
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse trancription
PCR	polymerase chain reaction
PP	peyers patches
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RIN	RNA integrity number
SCFA	short chain fatty acid
SEM	standard error of mean
Tab.	table
TGF-$ß_1$	transforming growth factor beta1
Th cells	helper T cells
TNF-α	tumor necrosis factor alpha
WBC	white blood cells

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ABSTRACT

The prebiotic lactulose is a non-digestible carbohydrate, which is metabolized in the colon by the saccharolytic microflora. Lactulose can be used to improve the intestinal microflora by stimulating the growth of probiotic bacteria in the gut and to compass an intestinal eubiosis. Furthermore, prebiotics and probiotics may enhance feed conversion and daily weight gain. The mechanisms by which lactulose affects growth performance, intestinal morphology and the immune system in pre-ruminant calves are not yet investigated in detail. This study was conducted to elucidate the effects of orally administered lactulose on probiotic-fed pre-ruminant calves.

Another study has been carried out, because low-quality RNA might strongly affect the experimental results of downstream applications. The particular problem of RNA degradation and its effect on relative quantification in qRT-PCR performance was tested in different bovine tissues and cell lines. Thus, gene expression changes by the use of degraded RNA in the physiological study were eliminated and future standards can be anticipated. Different artificial and standardized RNA degradation levels were used and the RNA quality was rated according to the RNA integrity number (RIN). A significant impact of RNA integrity on relative expression results, mainly on cycle threshold values and a minor effect on PCR efficiency was found. To minimize the interference of RNA integrity on relative quantification models a normalization of gene expression by an internal reference gene and an efficiency correction could recommend and were useful in the evaluation of the presented physiological study.

Calves were randomized into three feeding groups (n=14 each group) which received milk replacer containing *Enterococcus faecium* (*E. faecium*) supplemented with 0% lactulose (L0; control group), 1% lactulose (group L1) and 3% lactulose (L3 group). The calves were weighted weekly. After 19 weeks the calves were slaughtered and tissues were collected for histological and mRNA expression analysis. The main research of this doctoral thesis is the effect of lactulose on zootechnical performance as well as immunemodulatory effects. A broad spectrum of pro- and anti-inflammatory cytokines, T cell population markers, diverse lymphocytes activation markers and immunoglobulin A Fc Receptor (IgA FcR) in different immune and intestinal compartments were assessed. In addition, interactions among lactulose and the morphological development of the gastrointestinal tract (GIT), villi, crypts and lymph follicle in Peyer´s patches (PP) were determined. Changes in the intestinal mRNA gene expression of apoptotic markers were analyzed to confirm the histomorphometrical investigations.

The average daily live weight gain tended to be higher ($P<0.1$) for L3 (1350±59 g/d) than for L0 (1288±59 g/d), although a numerically larger difference was observed in male calves. Histomorphometric studies of the intestine showed various effects elicited by the lactulose rich feed in the ileum and caecum. Compared with L0, a reduction ($P<0.001$) of ileal villi height due to lactulose treatment and a decrease in the depth of the crypts in the caecum ($P<0.001$) was observed. In the caecum higher Caspase 3 ($P<0.05$) and TGF-ß$_1$ mRNA expression were detected. The surface area of lymph follicles from PP was decreased by lactulose treatment of female calves. Anti-inflammatory cytokines like IL-10 and TGF-ß$_1$ were up-regulated in several intestinal

compartments. The expression results indicated that the proportion of IgA FcR in the ileal mucosa of the 3% lactulose treated male calves increased and the IFN-γ mRNA expression decreased. $CD4^+$ presenting lymphocytes decreased in the ileum and mesenteric lymph node by 1% lactulose treatment of female calves, whereas $CD8^+$ presenting lymphocytes were increased in blood. The gene expression analysis of the anti-inflammatory cytokines showed an up-regulation of IL-10 and TGF-$ß_1$ in different compartments of the intestine.

The study results support the assumption that lactulose has an effect on the morphology of the intestine. A significant effect on growth performance can not be asserted. However, results permit the conclusion that lactulose feeding has the tendency to increase growth performance. The intestinal immune function may only be affected by the high dose treatment with lactulose, whereas the population of lymphocytes was more effected by 1% lactulose treatment. The results indicate that lactulose has a minor immune modulatory effect in different immune compartments and in the intestine. The effects of lactulose are obviously gender-specific: male calves tended to gain higher body weight and female calves tended to exhibit more changes in intestinal morphology. Our data suggest that the use of lactulose could be meaningful when calves have intestinal irritation. By comparing expenses and gain of performance the economic profit in raising calves might be questionable.

ZUSAMMENFASSUNG

Das Präbiotikum Laktulose gelangt unverändert in das Colon, wo es durch eine saccharolytische Mikroflora metabolisiert wird. Die Verabreichung von Laktulose dient der Wachstumsförderung probiotischer Bakterien und somit der Herstellung einer Eubiose im Darm. Prä- und Probiotika zeigen zudem die Fähigkeit, Leistungsparameter wie Futterverwertung und tägliche Gewichtzunahmen zu verbessern. Die Wirkung von Laktulose auf Wachstumsleistung, Darmmorphologie und Immunsystem preruminanter Kälber wurde noch nicht detailliert erforscht. Zur Aufklärung der Wirkung von oral verabreichter Laktulose in der kombinierten Anwendung mit dem Probiotikum *E. faecium* auf die Physiologie von preruminanten Kälbern wurde eine Studie durchgeführt.

Während der Doktorarbeit hat sich eine weitere Studie ergeben, da die Qualität der RNA die Auswertung der Genexpressionsanalysen stark beeinflussen kann. In einer methodischen Studie wurde die Fragestellung des Einflusses der RNA Qualität auf die relative Quantifizierung in der qRT-PCR aufgegriffen und mit unterschiedlichen bovinen Geweben und Zelllinien untersucht. Somit konnten Änderungen der Genexpression durch die Verwendung von degradierten RNA Proben in der physiologischen Studie ausgeschlossen werden. Aus einer RNA Probe wurden künstlich unterschiedliche Degradierungsstufen hergestellt und anschließend nach ihrer RNA Integritätsnummer (RIN) klassifiziert. Der Zyklusschwellenwert (Ct) bei der relativen Expression wurde stark beeinflusst, wobei die Effizienz der qRT-PCR nur gering beeinflusst wurde. Um den Einfluss der RNA Qualität auf die relativen Quantifizierungsmodelle zu minimieren, wird eine Normalisierung der Genexpression durch ein internes Referenzgen und eine Effizienzkorrektur empfohlen. Zur Auswertung der hier präsentierten physiologischen Studie wurden die neuen Erkenntnisse der methodischen Untersuchung angewendet.

Im Fütterungsversuch wurden 42 Kälber in drei homogenen Gruppen (n=14 pro Gruppe) eingeteilt. Die Kontrollgruppe (L0) wurde mit einem Milchaustauscher, der 10^9 Kolonie bildende Einheiten *E. faecium* enthielt, gefüttert. Zusätzlich zum Milchaustauscher wurde den Fütterungsgruppen L1 und L3 Laktulose in der Konzentration 1% (L1) bzw. 3% (L3) verabreicht. Die Kälber wurden wöchentlich gewogen und nach einer Versuchsdauer von 19 Wochen geschlachtet. Gewebe für die Histologie und Genexpressionsanalyse wurden bei der Schlachtung entnommen. Der Forschungsschwerpunkt dieser Doktorarbeit war die ertrags- und leistungsbezogene, sowie die immunmodulatorische Wirkung von Laktulose. Dazu wurden diverse pro- und antiinflammatorische Zytokine, T Zell Marker, Aktivierungsmarker der Lymphozyten und der Immunglobulin A Fc Rezeptor untersucht. Weiterhin wurde die Interaktion zwischen morphologischer Entwicklung des Gastrointestinaltraktes, sowie der Zotten, Krypten und Lymphfollikel in den Peyerschen Platten untersucht. Zur Klärung der Apoptoseregulation und zur Bestätigung der histomorphometrischen Untersuchungen wurden Vertreter der Bcl-2 Familie und weitere Apoptose- und Proliferationsfaktoren untersucht.

Die durchschnittlichen Tageszunahmen waren tendenziell höher in der Behandlungsgruppe L3 (1350±59 g/d) als in der Behandlungsgruppe L1 (1288±59 g/d), wobei die Differenz zwischen

beiden Behandlungsgruppen bei den männlichen Kälbern größerer war. In den darmmorphometrischen Studien via Histologie ergaben sich unterschiedliche Effekte der laktulosereichen Fütterung im Ileum und Caecum. Im Vergleich zur Kontrollgruppe L0 fanden sich bei den Laktulose gefütterten Kälbern reduzierte Zottenlängen im Ileum ($P<0.001$) und reduzierte Kryptentiefen im Caecum ($P<0.001$). Diese Ergebnisse konnten mittels Expressionsanalyse von diversen Apoptose- und Proliferationsfaktoren nur teilweise bestätigt werden. Im Caecum wiesen Caspase 3 und TGF-ß1 eine erhöhte mRNA Expression auf. Das Ausmessen der Lymphfollikel in den ilealen Peyerschen Platten ergab eine signifikante Abnahme der Follikelfläche (L1: $P<0.05$; L3: $P<0.01$) mit geschlechtsabhängiger Ausprägung. Antiinflammatorische Zytokine wie IL-10 und TGF-ß1 wurden in unterschiedlichen Darmabschnitten erhöht exprimiert. Weiterhin wurde durch Expressionsanalysen ein Anstieg der IgA FcR Expression und Abnahme der IFN-γ Expression im Ileum männlicher Kälber der Behandlungsgruppe L3 festgestellt. Bei weiblichen Kälbern wurden die CD4+ Lymphozyten im Ileum und im mesenterialen Lymphknoten runterreguliert, währenddessen die CD8+ Lymphozyten im Blut hochreguliert wurden.

Durch diese Ergebnisse wird deutlich, dass Laktulose eine Wirkung auf die Darmmorphologie hat. Einen signifikanten Effekt auf die Wachstumsleistung konnte nicht beobachtet werden, jedoch kann von einer tendenziell höheren Wachstumsleistung ausgegangen werden. Das Darmschleimhaut-immunsystem wird nur durch die hoch dosierte Laktulose Behandlung moduliert, wobei die Population der Lymphozyten nur durch eine niedrige Dosierung von 1% Laktulose verändert wird. Die Ergebnisse zeigen, dass Laktulose nur eine geringe immunmodulatorische Wirkung hat. Die Effekte sind geschlechtsspezifisch: männliche Kälber zeigen höhere Gewichtszunahmen und weibliche Kälber zeigen eher Änderungen der intestinalen Morphologie. Weitere Untersuchungen sind notwendig um auch die geschlechtsspezifischen Wirkungen eindeutiger zu beschreiben. Die Ergebnisse deuten darauf hin, dass die Fütterung von Laktulose bei einer Irritation der Darmschleimhaut sinnvoll sein kann. Fraglich ist jedoch bei einem Vergleich von Aufwand und Leistungssteigerung der ökonomische Vorteil in der Kälberaufzucht.

INTRODUCTION

The presented study is composed of a bioanalytical section and a physiological *in vivo* section. During the establishment of techniques a tissue dependent RNA integrity level was observed. It is well known, that an essential requirement for a successful quantitative mRNA analysis using qRT-PCR is the usage of intact RNA. Low-quality RNA may strongly affect the expression results. The knowledge about the influence of the RNA quality results in an intensive bioanalytical work, to eliminate errors in gene expression changes in the *in vivo* study.

Bioanalytics: problems with a successful quantitative mRNA analysis

The accuracy of gene expression evaluation is recognised to be influenced by the quantity and the quality of starting RNA. Investigations with low quality RNA as starting material supply wrong qRT-PCR results [1]. The most obvious problem concerns the degradation of the RNA and so the extraction and purification procedure of total RNA must fulfill several criteria such as: free of protein; free of genomic DNA; free of enzymatic inhibitors for Reverse Transcription (RT) and PCR reaction; free of nucleases for extended storage and the RNA should be undegraded [2,3]. There are a substantial quantity of problems that affect reproducibility and hence the relevance of results. The source of RNA, sampling technique, as well as RNA isolation techniques often vary significantly between processing laboratories [2,4]. The need to isolate high quality total RNA from wide variety of clinical and/or experimental tissue samples becomes more important for quantitative gene expression studies. Due to its inherent susceptibility to ubiquitous RNAses and its chemical instability, RNA is readily endangered by alkaline- or enzyme-catalyzed hydrolysis. After extraction the RNA is unstable and long mRNA, up to several kilo bases, is very sensitive to degradation [2]. Researchers must take into account a variety of factors, which influence their ability to obtain high-quality RNA that is free of contamination [5]. Verification of RNA integrity prior to usage in downstream qRT-PCR application permits experiments to be compared and provide more accurate and reliable results.

The micro-fluidic capillary electrophoresis, such as the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) has developed into a common tool for assessing the RNA quality, particularly in the gene expression profiling platforms [6]. RNA degradation is a gradual process with a decrease in the 28S to 18S ribosomal band ratio (Fig. 1). The 28S/18S ratio is calculated automatically, but it may not be used as a gold standard for assessing RNA integrity because of its high variability (coefficient of variability 19-32%) [1,5]. A new tool for a better interpretation of the RNA quality is the RNA Integrity Number (RIN). The algorithm assigns a RIN number scored from 1 to 10, where level 10 represents a completely intact RNA, and 1 presents a highly degraded RNA [6]. An interpretation of RNA integrity is facilitated, comparison of samples is possible and repeatability of experiments is ensured.

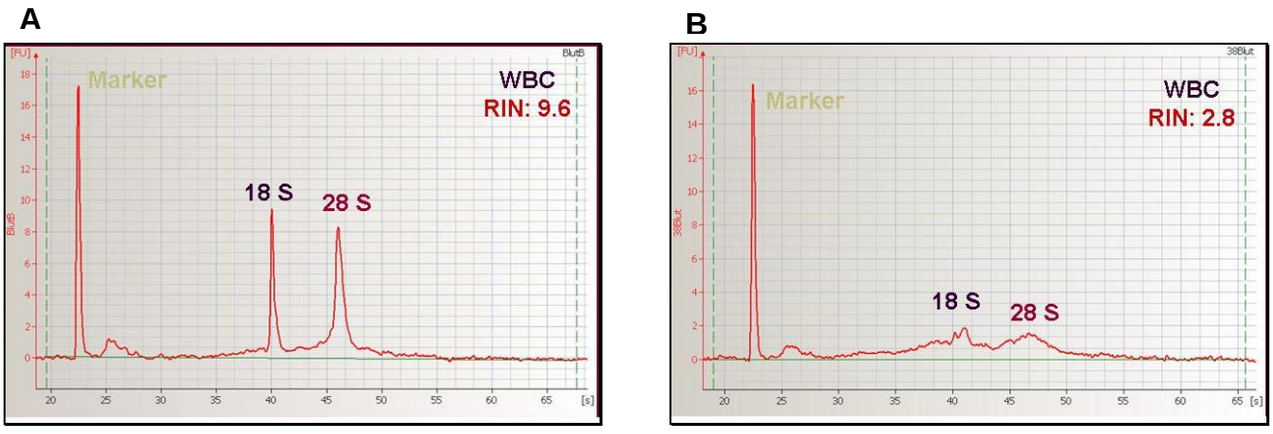


Figure 1: *Electropherograms from a white blood cell (WBC) sample with different RNA degradation levels, but with the identical transcriptome. A) No degradation of the RNA with a RNA integrity number (RIN) 9.6 and well formed 18S and 28S peaks. B) Highly degraded RNA with completely reduced 18S and 28S peaks which results in the RIN 2.8.*

While it is obvious that intact RNA constitutes the best representation of the natural state of the transcriptome, there are situations in which gene expression analysis even on partially degraded RNA may be desirable, e.g. in ancient, necrotic, clinical or biopsy samples. Nevertheless, little is known about the possibility of obtaining reasonable qRT-PCR data from RNA samples with impaired RNA quality. There are no statistical confirmed studies available at which threshold RNA integrity is useless for quantitative downstream applications.

In vivo investigations: Lactulose as feed additive

Prebiotics and probiotics as feed additive

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. Calves diseases – particularly diarrhea and respiratory diseases – have significant effects on the profitability of every calf raising enterprise. Young dairy calves are subjected to several stressors such as nutritional, environmental, social and microbial imbalances, which are the cause for disease. As a result, animals consume less milk [7], are predisposed to impaired intestinal morphology and intestinal function [8,9], showed a high incidence of diarrhea and growth depression and may be afflicted with impaired immune function [10].

For several decades, prophylactic doses of antibiotics and chemotherapeutics have been used in animal feed to improve animal welfare and to obtain economic benefits in terms of improved animal performance and reduced medication costs. However, there are increasing concerns about the risk of developing multiple antibiotic resistances in pathogenic bacteria and cross-resistance in livestock. With the ban on dietary antibiotics as growth promoters within the European Union,

animal nutritionists are seeking alternatives to these promoters, particularly for young animals [11]. Potential feed additives in the diet for young calves to date are prebiotics, probiotics, vitamins, minerals [12] and organic acids.

In the first instance the maintenance of the gut health is an important objective. One of the approaches for enhancing the beneficial endogenous microflora in the gut is the use of prebiotics. The term *prebiotic* was introduced by Gibson and Roberfroid [13] who defined prebiotics as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon." This definition more or less overlaps with the definition of dietary fiber, with the exception of its selectivity for certain species. This selectivity was shown for bifidobacteria, which may be promoted by the ingestion of substances, mostly oligosaccharides (OS), such as fructo-OS and inulin [13,14,15], transgalactosylated-OS [16], galacto-OS, soybean-OS [17], isomalto-OS and oligofructose (Tab. 1).

Physiological effects of non absorbable carbohydrates include increased fecal bulk, increased short chain fatty acids (SCFA) production, and modification of bacterial populations [18,19]. They beneficially affect the host by selectively stimulating growth and /or the activity of one or a limited number of bacteria in the colon [13,20,21]. Prebiotics that stimulate the intestinal growth of probiotics lactobacilli include several well-characterized OS [22], inulin and lactulose [18,23,24]. It is expected that prebiotics will improve health in a way similar to probiotics, whilst at the same time being cheaper, carrying less risk and being easier to incorporate into the diet than probiotics [25].

Table 1: *Major oligosaccharide candidates for prebiotics*

Oligosaccharides (OS)	Structure	Linkages	Process	Origin
Xylo-OS	(Glu)n	ß-1,4	Hydrolysis	Cereals
Lactulose	Gal-Fru	ß-1,4	Isomerisation	Lactose
Isomalto-OS	(Glu)n	α-1,6	Hydrolysis	Algae
Gluco-OS	(Glu)n	α-1,2 and α-1,6	Synthesis	Sucrose
Galacto-OS	(Gal)n-Glu	ß-1,4 and ß -1,6	Synthesis	Lactose
Fructo-OS	(Fru)n-Glu	(ß-2,1)- α-1,2	Synthesis	Sucrose
Oligofructose	((Fru)n-(Fru)n-Glu	(ß-2,1)	Hydrolysis	Inulin

Lactulose is a disaccharide analogue of lactose that cannot be digested by mammalian enzymes because of its specific structure (4-O-ß-D-galactopyranosyl-D-fructose) (Fig. 2). Thus, lactulose cannot be absorbed by the small intestine nor digested by pathogenic bacteria, but it is a suitable substrate for some probiotic bacteria in the gut [26]. As such, lactulose is a tool for regulation of the bacterial ecology [27].

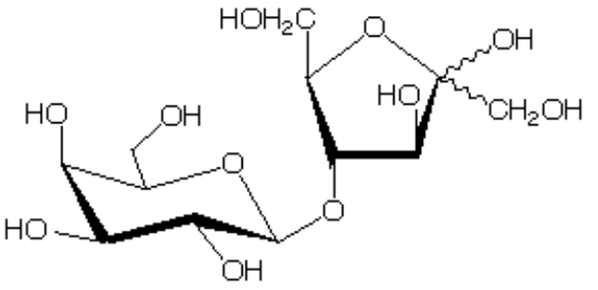


Figure 2: *Chemical structure of Lactulose*

Numerous scientists investigated the health-promoting effect of prebiotics like indigestible sugars, e.g. fructo-OS, inulin and lactulose [15,28]. The positive effects of lactulose on colonic metabolism in human, rat, mouse, and pig are well known [29,30]. Several reports have shown that supplementing a diet with oligofructose or lactulose improved growth in pigs [31,32] or in calves [33]. Other reports did not find growth effects [34,35]. The reasons for the different results may be due to the different chemical structure and compositions of the prebiotics used. Other factors which may also explain variations in results include growth phase of the animal, the type of dosing used and the hygienic condition of the housing [36].

Furthermore, probiotics may be used as one of the potential alternatives for feed additives. The term is used to describe living microbial feed supplements which beneficially affect the host animal by improving its microbial balance [37,21]. They have been reported to increase feed intake, growth rate, weight gain, improve feed conversion, decrease fecal coliform count, reduce demand for antibiotic treatment [38] and enhance immune responses [39]. There are several proposed mechanisms by which probiotics may protect the host from the intestinal disorder [40]:

- Probiotics produce several inhibitory substances such as organic acids, hydrogen peroxide and bacteriocins. These substances may limit the harmful bacteria in the gut.
- Probiotic microorganisms may prevent the utilization of nutrients by pathogenic bacteria.
- Competitive inhibition for pathogen bacteria adhesion on intestinal epithelial surfaces which will allow to rapidly colonization of the intestinal trac.
- They can protect against intestinal disease by stimulating the specific and nonspecific immunity.

The aerobic, gram-positive lactic acid bacteria *Enterococcus faecium* (*E. faecium*) is a normal inhabitant of the gut flora of animals [41] with inhibitory effects against important enteropathogens [42]. It was recently shown, that E*. faecium* stimulates the mucosal and the systemic immune system in dogs [43]. Furthermore *E. faecium* stabilizes the intestinal microflora and increases the weight gain of calves and young pigs [44,45].

Another possibility of microflora management is the use of synbiotics, whereby probiotics and prebiotics are used in combination [13]. The synbiotics will have more beneficial effects on gut health and growth than using either pre- or probiotics separately, because the synergistic effects

can be useful in stimulating beneficial bacteria and improving the health of the gut [40]. The effects of a synbiotic feeding on the immune response were analyzed in previous studies with rats [46], mice [47], dogs [48] or pigs. Furthermore, results of several studies have been inconsistent and thus they were not directly comparable. However, there is little information on synbiotics and its possible mechanisms in young calves.

Intestinal immune system

The gut and the immune system have evolved to provide effective digestion and simultaneously defense against ingested toxins and pathogenic bacteria. Most protection against potential pathogens occurs via a mucosal immune system involving mechanisms of innate immunity as well as a secondary lymphoid organ, the gut-associated lymphoid tissue (GALT) [11]. The unique characteristics of the intestinal immune responses are secretory immunoglobulin A (IgA) production and oral tolerance [47]. The ability of the mucosal immune system to distinguish between harmful and harmless antigens is essential to mount a protective immune response and to prevent the induction of mucosal pathology [49]. Oral tolerance presumably evolved to prevent hypersensitivity reactions to food proteins and bacterial antigens in the mucosal microbiota [50].
The regulatory events of the intestinal immune response occur in different physiologic compartments: aggregated in follicles and peyers patches (PP) and distributed within the mucosa, the intestinal epithelium, and secretory sites [51]. The inductive site of the immune reactions to oral antigens, mainly the PP consists of aggregations of lymphoid follicles. Their surface is covered by the follicle-associates epithelium which contains specialized epithelial cell types, known as M cells. The M cells transport antigens via transcytosis from the lumen to cells of the immune system, thereby initiating an immune response or tolerance [52]. In the PP, antigens are presented by antigen presenting cells (macrophages and dendritic cells) to both immature T and B cells [53]. Activated T cells preferentially differentiate into $CD4^+$ (cluster of differentiation) T helper cells which, aided by dendritic cells and secretion of cytokines such as transforming growth factor beta 1 (TGF-β_1) and interleukin 10 (IL-10), induce the differentiation of antigen-specific B cells to predominantly IgA-committed plasmablasts [54,55,56]. Early studies suggested that $CD8^+$ "suppressor" T cells were important, but it is now accepted that it may involve either anergy/deletion of $CD4^+$ T cells, or the induction of regulatory $CD4^+$ T cells that produce IL-10 and/or TGF-$ß_1$ [57]. The GALT derived B cell blasts proliferate and differentiate further on their way through the mesenteric lymph nodes (mLN) and the thoracic duct into the bloodstream. Then they migrate preferentially to the mucosal effectors site (i.e. lamina propria and intraepithelial regions, but not PP). Here they complete their terminal differentiation to IgA-producing plasma cells – a process called ‘homing’ [53]. A schematic depiction of the functional organization of the GALT with inductive and effector sites, is shown in figure 3.

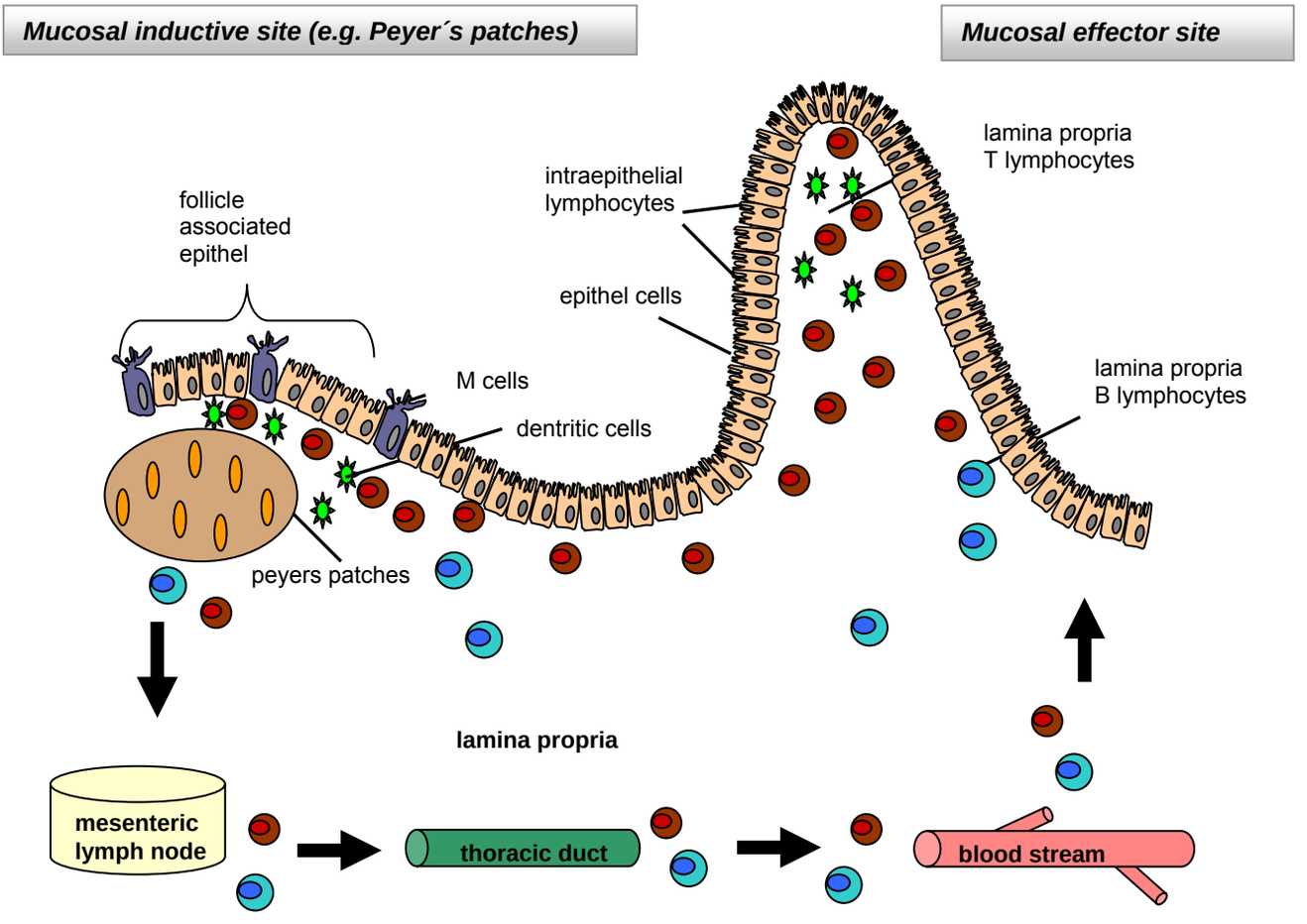


Figure 3: *Schematic functional organization of the GALT, which is divided into inductive (PP) and effector sites (lamina propria). Antigen transport across the epithelium occurs through M cells and dendritic cells. After being primed in the GALT, B and T cells differentiate further on their way through the lymph nodes and the blood and migrate to the mucosal effector sites [58,59].*

Stimulation of the immunity by probiotics and prebiotics

The beneficial effects of bacteria on the immune system have been proposed as one theory supporting the use of probiotic bacteria as an alternative to antibiotics in improving animal health and protection against infectious agents [60]. Probiotic supplements induce immunological responses in the host [11,39,40,61], and prebiotics like lactulose stimulate the growth of selected intestinal microflora [13,20,21]. Many probiotic effects are mediated through immune regulation and stimulation, such as enhanced antibody response [62] and particularly by regulation of cytokine production [61]. They mediate suppression of lymphocyte proliferation and cytokine production by T cells [62] and down-regulate the expression of pro-inflammatory cytokines as TNF-α, IL-1ß and INF-γ [63]. They interact with intestinal epithelia, and reduce synthesis of inflammatory effector molecules elicited by diverse pro-inflammatory stimuli [64,65]. That means probiotics can be responsible for the unique tolerance of the gastrointestinal mucosa to pro-inflammatory stimuli. At

present less prebiotic substances are known that beneficially affect the immune system [48]. It was reported that inulin and oligofructose can modulate functions of the immune system, primarily the activation of immune cells in PP [66]. The simultaneous application of lactulose and the probiotic bacteria *E. faecium* could amplify the beneficial effects on the immune system.

AIM OF THE STUDY

The intactness of RNA and its influence on the qRT-PCR results from different bovine tissues and cell lines were analyzed to count out gene expression changes in the presented *in vivo* study by the use of degraded RNA and to anticipate future standards for the threshold at which RNA integrity is useless for quantitative down-stream applications. Furthermore, the correlation between RNA quality, PCR performance, PCR efficiency and length of amplified product was analyzed.
The major objective of the *in vivo* study was to investigate the mode of action of lactulose on the intestinal immune function, intestinal morphology, growth performance and health of *E. faecium* fed pre-ruminant calves.

MATERIAL AND METHODS

Bioanalytical investigations: RNA quality and the effect on qRT-PCR results

Sample preparation and artificial total RNA degradation

Two experiments have been carried out. The particular problem of RNA integrity and its effect on relative quantification in qRT-PCR performance (experiment 1) was tested in different bovine tissues and cell lines (n=11). The effect of different length of amplified products and RNA integrity on expression analyses was investigated in the second experiment with extracted RNA from four bovine tissues. RNA purification in both experiments was performed by an in-house standardized phenol-based extraction method using peqGOLD TriFast (PeqLab, Erlangen, Germany) [72]. In order to get RNA samples with different and standardized degradation levels, but with the identical transcriptome and tissue typical mRNA distribution, intact cellular RNA was artificially degraded either enzymatically via ubiquitary skin RNAses or by irradiation with ultraviolet-C radiation at 200-280 nm (Kendro Uv-C30, Langenselbold, Germany). For both experiments, intact RNA samples from the identical tissue extraction were mixed in various ratios with degraded RNA samples to generate a RIN gradient. Each sample consist of 10-12 denaturation grades and ranging from intact RNA (RIN>7.5) to highly degraded RNA (RIN<3). In total 135 samples were investigated. For the analysis different artificial and standardized RNA degradation levels were used and the RNA quality was rated according to the RIN [72]. For the rapid quantification of nucleic acids the Agilent 2100 Bioanalyzer (Agilent Technologies) was used.

One step qRT-PCR and relative quantification

In first experiment the expression levels of four representative genes (18S, 28S and ß-actin and IL-1ß) were measured. High abundant ribosomal 18S and 28S rRNA subunits, medium abundant ß-actin and low abundant IL-1ß genes were used to cover all possible abundance levels of a normal distributed transcriptome. Quantification was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA) [72]. Ct values and qPCR efficiency were computed with the "*comparative quantification"* method in the Rotor-Gene 3000 software version 6.0 (Corbett Life Science, Sydney, Australia). In the first relative quantification approach no efficiency correction was performed according to the delta-delta Ct model [67]. In the second approach the efficiency correction was implemented additionally (Fig. 4) and the advances of an efficiency corrected quantification model were used [73]. The derived relative expression values describe the relative quantification change in expression of the target gene relative to the reference gene 28S expression.

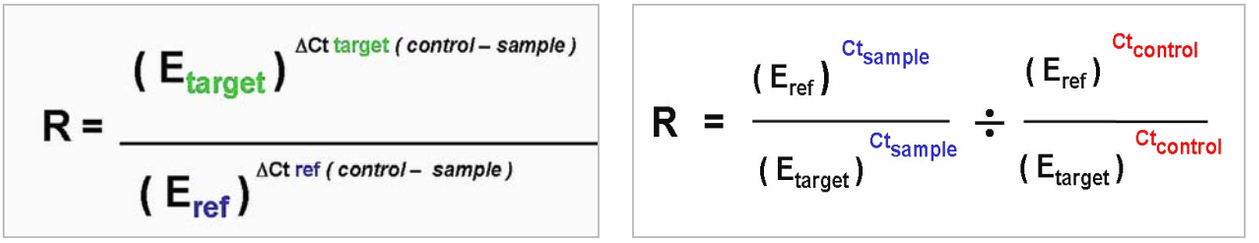


Figure 4: *Efficiency corrected quantification model*

For the second experiment seven PCR primer sets were designed using HUSAR software (DKFZ, Heidelberg, Germany) and synthesized by MWG Biotech (Ebersberg, Germany) to amplify different sequence fragments of ß-actin [72]. All statistical analyses were performed by using Sigma Stat 3.0 (SPSS Inc., Chicago, IL). The determined *P*-values were analyzed using linear regression and the coefficient of determination (R^2). Significance of linear regression was tested by student t-test.

In vivo investigation: Physiological effect of lactulose in calves

Animals, husbandry, feeding and experimental procedures

42 Simmental calves were divided into three homogenous experimental groups (n=14) according to their weight, age and gender (7 male and 7 female calves) as previously described [68]. Animals were housed at the experimental station Karolinenfeld (Bayerische Landesanstalt für Landwirtschaft - LfL, Institute of animal nutrition and feed economy). During the feeding experiment, all calves were fed with milk replacer (MR) from Milkibeef Top (Milkivit, Trouw Nutrition, Burgheim, Germany) added with 10^9 CFU *E.faecium* per kg MR. Feeding group L0 served as control. The other two groups were fed with MR enriched by 1% (L1) and 3% (L3) dry matter lactulose (Lactusat, Milei GmbH, Germany). 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/d 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 ± 8.3 days, animals were slaughtered. The general health status of the calves was monitored by daily physical examination, checking general appearance, animal activity, feces composition, and time to time rectal temperature. Animals were further inspected by a veterinarian on a weekly basis to confirm identical health status of the feeding groups. The experimental procedures followed the current German law on animal production and veterinary inspection (LfL, Grub, Germany).

The statistical analysis of the zootechnical performance was done by Sigma Stat 3.0 (SPSS Inc.) using the two-way ANOVA.

Tissue collection and blood samples

***Tissue sampling*:** The GIT was removed and a 1- to 2 cm long cross-section from the small intestine (mid jejunum, mid ileum), large intestine (caecum, colon), mLN and spleen were obtained at the time of slaughter. Immediately after collection the tissues were placed into individually labeled cryotubes and frozen in liquid nitrogen. The cryotubes were stored at -80°C until analysis [69]. For histological analysis the tissue pieces were washed twice in physiological 0.9% NaCl solution and the tissue sections were embedded and transferred in 10% buffered formalin (Carl Roth GmbH, Karlsruhe) for 24h as described previously [68,79].

***Blood sampling*:** 2x 15 ml blood samples were collected from the jugular vein using an EDTA and serum vacutainer tube (Greiner bio-one GmbH, Frickenhausen, Germany) following euthanasia. For hematological analysis the blood samples were forwarded in EDTA vacutainer tubes to a veterinary laboratory (Vetmed Labor, Unterhaching, Germany). Hematocrit and hemoglobin concentration, erythrocyte, thrombocyte and white blood cell (WBC) numbers were determined with the CELL-DYN 3700SL System (Abbott Diagnostika GmbH, Wiesbaden, Germany). Furthermore a blood smear was sent to obtain a differential white cell count. Blood smears were stained with May-Grünwald's eosin-methylene blue solution (Merck 1424) and Giemsa solution (Merck 9204) [70]. The percentage of the different WBC subtypes, like granulocytes, lymphocytes, monocytes and atypical cells were determined. For WBC mRNA expression analysis EDTA blood was diluted 1/1 (v/v) with lysis buffer (830 mg NH_4Cl, 3.7 mg Na-EDTA, 100 mg KCl in 100 ml H_2O pH 7.4) and centrifuged for 10 min at 220 g. The cell pellet was again suspended in lysis buffer and centrifugation was repeated. Supernatants of blood samples were discarded and leucocytes were transferred in 350 µl RNA extraction lysis buffer (Macherery-Nagel, Düren, Germany).

Histology and histomorphometry of intestinal mucosa

The collected specimen were trimmed and embedded in paraffin. Thin sections (7-8 µm) were cut using the Microtom LEICA RM2145 (Leica, Wetzlar, Germany), mounted on glass slides, and stained with haematoxylin and eosin according to Mayer (1969) and covered with Euktit (Merck, Darmstadt, Germany). Histological sections were examined with the light microscope Axioskop 2 plus (Zeiss, Oberkochen, Germany) with a 10x/0.30 Plan-Neofluar objective connected to the video-based, computer-linked AxioVision 3.1 system that was programmed to perform morphometrical analysis [71]. Only for the measurement of the lymph follicle in the PP the Stemi 2000-C (Zeiss) was used with the 2.5 x objective. Pictures were taken with the AxioCam MRc (Zeiss). The applied objective was changed depending on the examined tissue.

Villus height, crypts depth and the width were evaluated on three well-orientated villi- and crypt-preparations for each intestinal sampling site (Fig. 5). Triplicate measurements for every category (height, depth, width) and section (jejunum, ileum, caecum, colon) were evaluated. Furthermore, the area of at least six lymph follicles of PP in the ileum (n=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.

All measurement values are expressed as mean ± standard error of mean (SEM). For group differences, villus heights and width, villus height/width ratios, crypt depths and width and surface of the lymph follicle of PP were analyzed with the statistical program Sigma Stat 3.0 (SPSS Inc.) using two-way ANOVA. In order to find out whether lactulose has different sex-specific effects the pair wise multiple comparison procedures were processed with the Holm-Sidak method. The significance level was set at 0.05 for all tests.

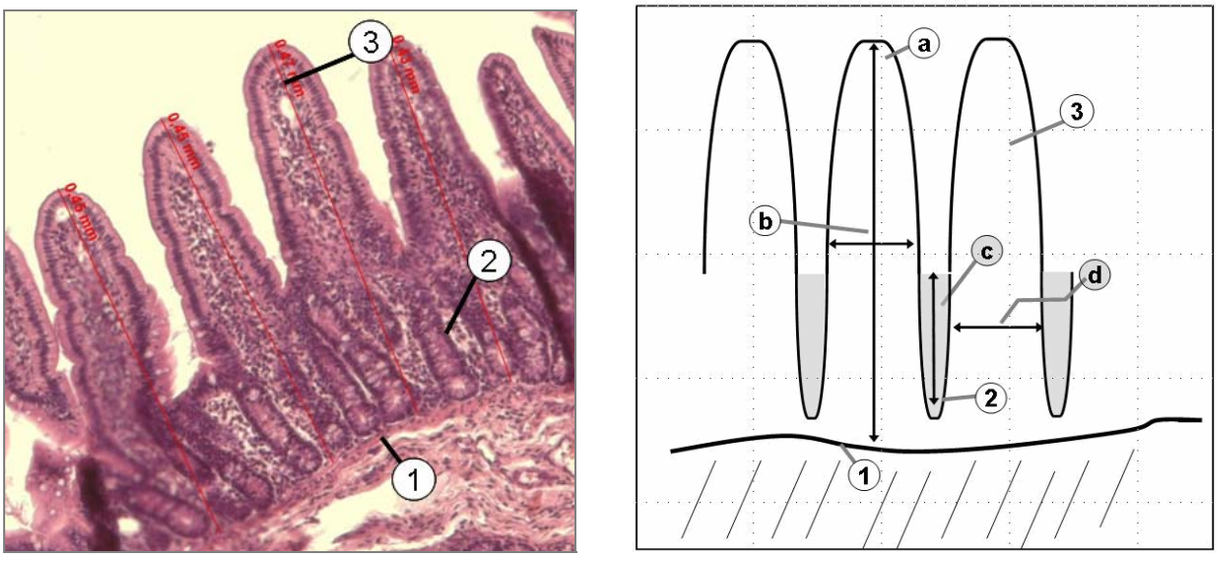


Figure 5: *Morphological measurements in the intestine: (1) lamina muscularis mucosae; (2) crypt of Lieberkuhn; (3) villus. Measurements in the small and large intestine were combined pictured in the diagram. Small intestine: (a) villus height (from the tip of the villus to the lamina muscularis mucusae; (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).*

Total RNA extraction and mRNA analysis

Total RNA from blood samples and each tissue sample (~50 mg) was extracted by using TriFast reagent (Peqlab) essential 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 A_{260} nm/A_{280} nm absorption ratio. A second quality control was done by a micro-fluidic capillary electrophoresis [72]. 100 ng of each experimental RNA sample was loaded onto a RNA 6000 Nano Chip and examined using the Bioanalyzer 2100 (Agilent

Technologies). A commercially available and optimized RNA length standard ladder (Ambion, Austin, TX) during electrophoresis allows the evaluation of sizes of RNA bands.
One step real-time qRT-PCR was performed by using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen Life Technologies) by a standard protocol in a Rotor-Gene 3000 (Corbett Life Science) as described previously [69]. Bovine sequence-specific primers were designed by using the HUSAR program (DKFZ) and were synthesized by a commercial facility (MWG Biotech). Sequences used for primer design were obtained from public databases (GenBank, National Center for Biotechnology Information) as coding DNA sequence. The primer sequences are listed in table 2.

Data evaluation

Cycle threshold (Ct) and single run efficiency (E) values were achieved for each analyzed sample and gene using the Rotor-Gene 3000 software version 6.0 (Corbett Life Science). Ct and E data for male and female calves (each n=7) were analyzed separately. The relative expression changes were evaluated by using the single-run-specific efficiency-corrected relative expression model [73]. But in contrast to the previous described model (Fig 4), the advanced relative quantification model was used.
To assess the effect of the lactulose feeding 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 (ß-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 6.

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

Figure 6: *Single-run-specific efficiency-corrected relative expression. For normalization of each analyzed target gene in the presented study the mean expression of three reference genes (ß-Actin, GAPDH, Ubiquitin) was calculated. Further the PCR efficiency of mentioned genes was included in the calculation model.*

Cytokine mRNA expression is presented as relative expression whereas the appropriate mean control group value was used as the calibrator. Values were expressed as means ± SEM. Analysis for cytokine expression was done by the two-way ANOVA. In order to find out whether lactulose has different sex-specific effects the pairwise multiple comparison procedures were processed with

Holm-Sidak method. The significant level was set at 0.05 for all tests and the statistical analysis was performed with Sigma Stat 3.0 (SPSS Inc.).

Table 2: *Primer used for qRT-PCR.*

Identity	**Sequence [5'→ 3']**		**Size [bp]**	**Accesion No**
ß-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		
Ubiquitin	FOR	AGATCCAGGATAAGGAAGGCAT	198	Z18245
	REV	GCTCCACCTCCAGGGTGAT		
IL-1ß	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-$ß_1$	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		
INF-γ	FOR	CTT GAA TGG CAG CTC TGA GAA AC	112	M29867
	REV	GGC CTC GAA AGA GAT TCT GAC		
IgA FcR (CD89)	FOR	GAC AAA CCC TTT CTC TCC ACC	180	AY247821
	REV	ACA GGA CCC AGA GTG AAG TC		
CD25	FOR	ATG GAG CCA AGC TTG CTG ATG T	171	Multi
	REV	TCT GCG GAA GCC TGT CTT GCA		
CD69	FOR	GTC ATT GAT TCT AAA GAG GAC ATG A	137	AF272828
	REV	AGG TTG AAC CAG TTG TTA AAT TCT		
$CD4^+$	FOR	GAT CGA GGT CTT GCC TTC AG	237	Multi
	REV	GAT CTG AGA CAT CCG TTC TGC		
$CD8^+$	FOR	ACT GTG TAT GGC AAG GAG GTG	127	XM585436
	REV	GGG TAT CCC AAT GAT CAT GCA G		
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		

RESULTS AND DISCUSSION

In the bioanalytical research the intactness of RNA and its influence on the qPCR results were analyzed. Future standard for the work with degraded RNA should elaborate and the correlation between RNA quality, PCR performance, PCR efficiency and length of amplified product was specified. The new acknowledgements were used for the evaluation of the *in vivo* study with calves. Consequently gene expression changes by different output RNA integrity in the *in vivo* study were count out.

Bioanalytical research

Many factors present in samples as well as exogenous contamination have been shown to inhibit RT as well as PCR. Further problem may arise in the case of research on human or animal tissue sampling techniques and the time dependency until the tissue is stored safely in RNase inhibitors or RNA-later. It is often very challenging to decrease this sampling time to a minimum within the framework of clinical routine procedures, or in animal experiments during a slaughtering process. The dependence of the RNA integrity on various calf tissue samples, WBC and different cell lines was determined in this presented work (Fig. 7). As shown for solid tissues the average RIN is between 6 and 8. Tissues or organs with high content of connecting tissue, e.g. in the gastrointestinal tract like rumen, omasum and jejunum, underlie high RNA degradation through the sampling and extraction procedure and show great RIN variations. The reason for this variability might be the solid and tough structure of the tissues, e.g. connecting or fatty tissue, the RNase enzymatic activity and problems during tissue sampling and storage. Furthermore tissues from the gastrointestinal tract have been washed in physical saline solution to get rid of any disturbing gut substances. Thus the physiological constitution of the tissue, the time and management of tissue sampling has a bearing on the degradation level of RNA. In contrary single cells like WBC or cell derived from cell-lines have higher RIN. Cell sampling and RNA extraction is much faster and easier, because cells are better accessible and were not kept in any sub-optimal conditions. Therefore RNA integrity based on the RIN classification is much better and lay around RIN 9. The importance of isolation technique for a good RNA quality is shown in detail in Fleige *et al*. [72].

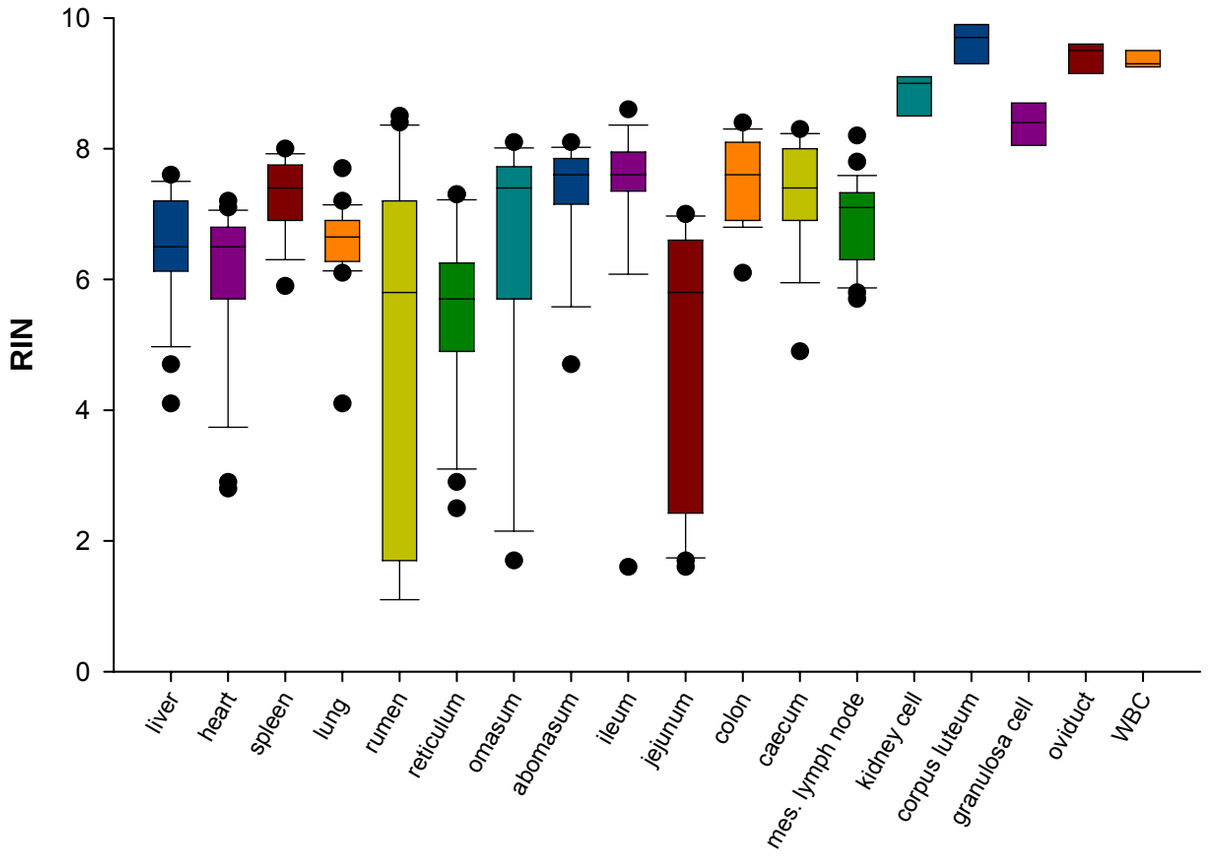


Figure 7: *Average RIN of various bovine tissues and cell lines. The bovine tissues and the WBC derived from the presented in vivo study with calves. Solid tissues showed an average RIN between 6 and 8, whereas tissues from the gastrointestinal tract like rumen, omasum and jejunum show great RIN variations. High quality RNA was achieved from WBC or cells derived from cell-lines (modified by Fleige, 2006).*

Effect of RNA integrity on PCR performance

Each amplified gene was tissue specifically influenced by the RNA integrity. This demonstrates an incomprehensible tissue-matrix-effect between RNA integrity and type of tissue and the analyzed transcript (Fig. 8A). A significant negative relationship between the RIN and Ct for all tested samples is proven (P<0.001). Other reports could confirm the direct influence of RNA integrity on the absolute expression results [1]. Degradation does not prelude micro array analysis if comparison is done using samples of comparable RNA integrity [74]. The effect of RIN on PCR efficiency was investigated similarly to the above mentioned tissues and various RNA qualities [5,72]. The efficiency of all investigated genes was not affected by the RNA quality, independent of gene or tissue (Fig. 8B). Some tissues reveal a positive coefficient of regression, but looking over all analyzed genes and tissues [72] the RIN effect on qPCR efficiency was minor, compared to the influence on Ct.

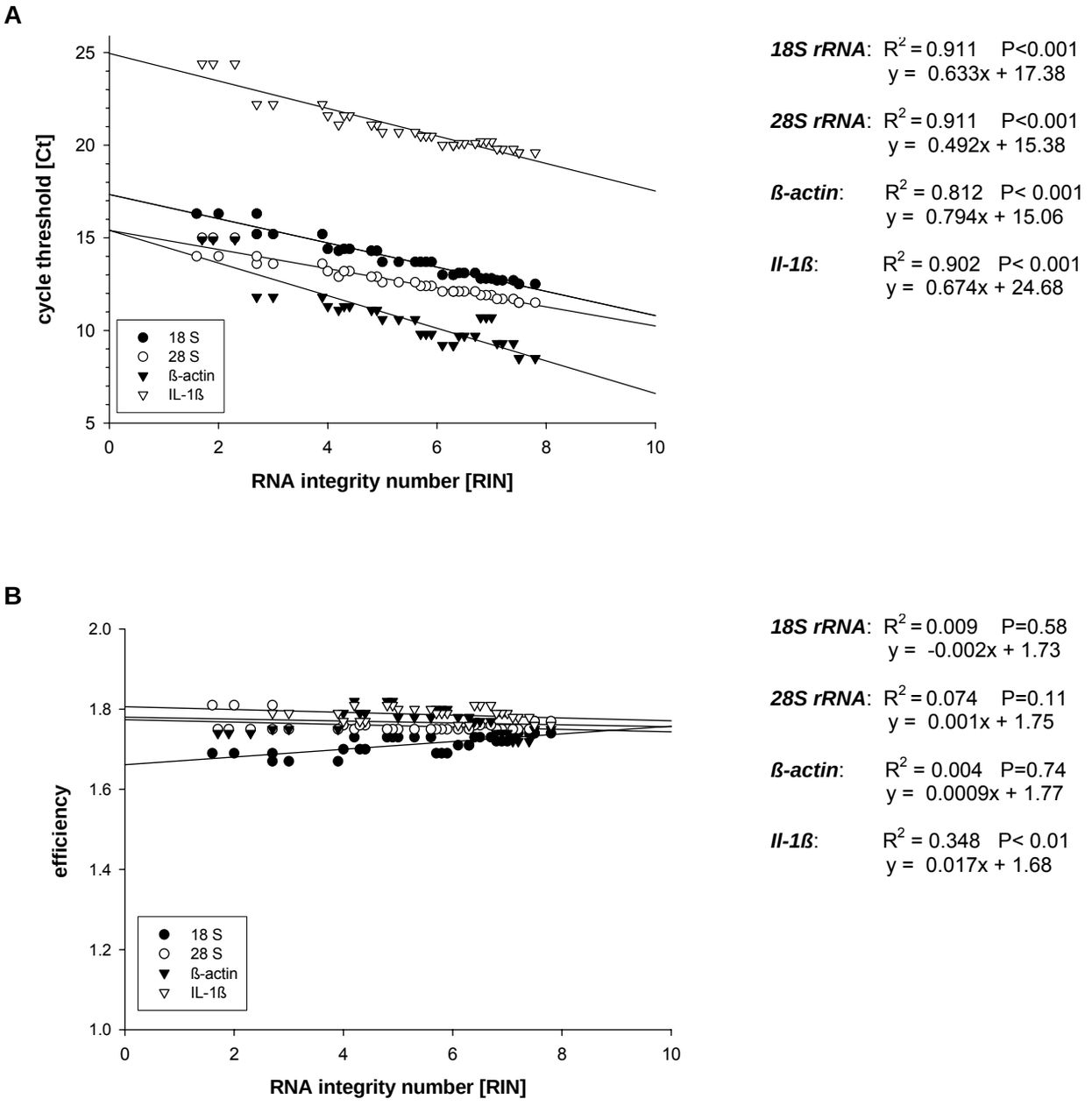


Figure 8: *Distribution of the qRT-PCR absolute expression measured on 12 RNA aliquots in triplicates (n=36) from lymph node (cited as an example for all tested tissues and cell cultures) using 18S, 28S, ß-actin and IL-1ß. (A) Negative correlation between cycle threshold and RIN. (B) No correlation between single-run PCR efficiency and RIN.*

Impact of RIN on relative quantification

Normalization by an internal reference gene reduces or even diminishes tissue derived effects on qRT-PCR [75]. To test the effect of normalization, expression results of 18S, ß-actin and IL1ß were normalized to the reference gene 28S rRNA. The applied standard should be not regulated or be at least minor regulated, like in the applied study the 28S rRNA expression. An accurate and relevant

normalization to some internal standard is obligatory for biologically meaningful mRNA quantification [76]. The relative quantification alteration in expression of the target gene relative to the reference gene was performed in two ways: first according to the delta-delta Ct method, and second by the single-run-specific efficiency-corrected relative expression model. Normalization of expression data on the basis of varying RNA integrities with an internal reference gene resulted in both models a RIN dependent effect [5,72]. For nearly all genes and tissues we could show a significant effect on relative expression level dependent on the RNA quality (P<0.001). To visualize the results, as shown after normalization, fourfold more ß-actin molecules could be found comparing highly intact to degraded RNA (Fig. 9), meaning around 75% ß-actin mRNA was degraded. Statements about importance of normalization and efficiency correction could certify herewith. Results demonstrate that innovative new quantification methods and models can improve future mRNA quantification.

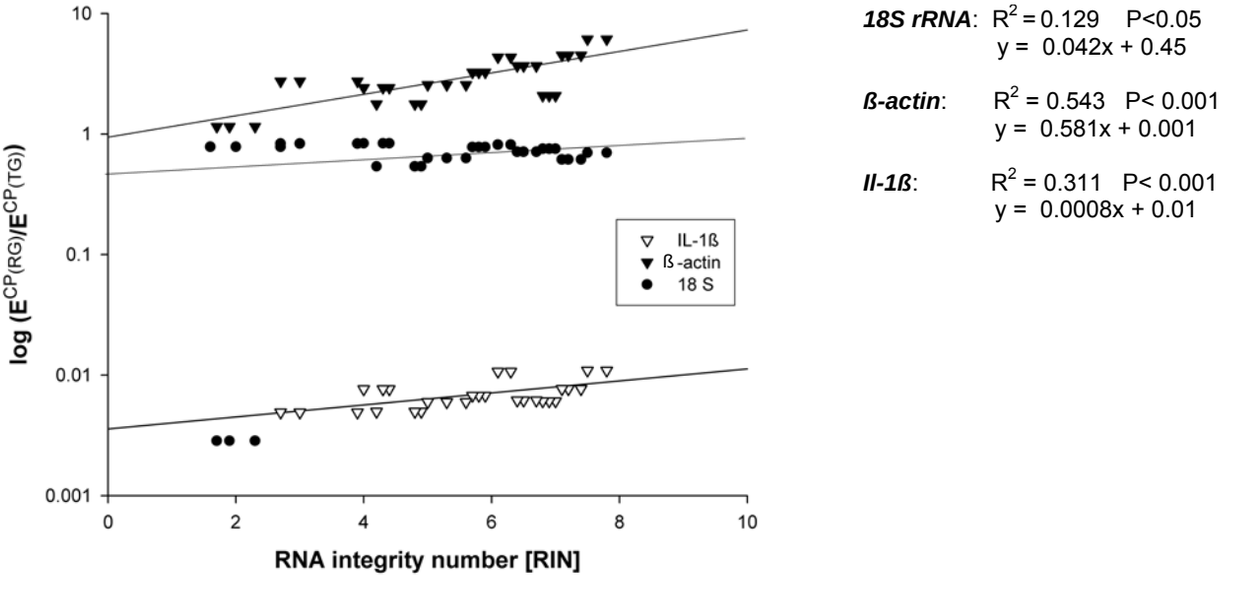


Figure 9: *Single-run efficiency-corrected relative expression ratio $E_{(RG)}$^$Ct(RG)$ / $E_{(TG)}$^$Ct(TG)$ ratio versus RIN. Distribution measured on 12 RNA aliquots in triplicates (n=36) from lymph node (cited as an example for all tested tissues and cell cultures) using 28S as reference gene. All genes showed a significant effect on relative expression levels dependent on the RNA quality (P<0.001).*

Interrelation between RIN and length of amplified product

The length of the amplified product influences the PCR efficiency such as primer length, annealing temperature, and secondary structure [2]. Quantitative RT-PCR involves analysis of smaller mRNA regions and is therefore more tolerant of partially degraded RNA. To test the effect of length of amplified PCR products on qRT-PCR, a gradient with several steps of intact RNA down to degraded RNA was examined with seven primer sets, amplifying qRT-PCR products of various lengths up to 976 bp. The results of correlation between RIN and Ct fulfilled the expectation for all

tested tissues that with increasing RIN the Ct is shifted to lower cycle number for all product lengths. Similarly, low quality RNA pointed a high variability in qRT-PCR results (Fig. 10). The graph is subdivided in four quadrants, which show the best repeatability in quadrant IV with high RNA integrity (RIN>5) and product length up to 200 bp. These results could be confirmed for all analyzed tissues. Quadrant I and III showed low RNA quality (RIN<5) and high variability in qRT-PCR results. Late and highly variable Ct values (quadrant II) are also due to amplified product length over 400 bp and good RNA quality. High Ct values for 800 and 950 bp may result form inefficient amplification as from to long qRT-PCR products and the applied stringent cycle conditions. Maximum amplicon size should not exceed 400 bp (ideally 80-150 bases). Smaller amplicons give more consistent results because PCR is more efficient and more tolerant in reaction conditions. The length of the amplified product is a very important part for primer design.

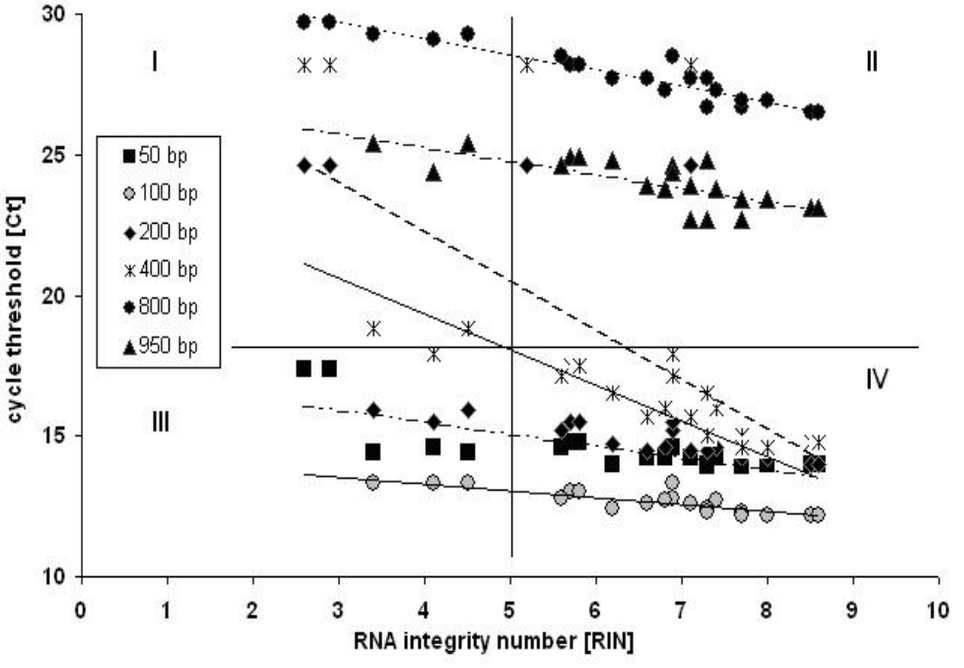


Figure 10: *Relationship between RNA integrity and length of the amplified product. Integrity of 23 bovine corpus luteum RNA sample (cited as an example for all tested tissues) profiles was scored using the RIN software. Cycle threshold (Ct) values in dependence on amplicon length and RNA integrity. Comparative analysis was done using ß-actin with different length of the amplified product and the graph is divided in four quadrants (I–IV).*

The new acknowledgements about the influence of different RNA quality on the PCR performance were used for the evaluation of the *in vivo* study. All gene expression analyses in the *in vivo* study were done by the single-run efficiency-corrected relative expression model. Consequently gene expression changes by different output RNA integrity in the *in vivo* study were minimized.

Physiological effect of lactulose in calves

The work on probiotics for cattle has increased in recent years and usually has shown a beneficial effect on the host. Thus we used a MR containing the probiotic bacteria *E. faecium* to achieve a possible improvement in health. In the last ten years, positive 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 [38].

Feed intake and zootechnical performance

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. The present results in table 3 demonstrate the effects of lactulose on feed intake and growth performance [68]. The average daily MR intake was significantly higher in feeding group L3 (P<0.05). An increased intake of crude protein and energy for group L3 was achieved, due to the feeding of Lactusat (Milei). Male calves showed a similar average daily MR intake between treatment groups, though the female calves of group L1 showed a lower average daily MR intake (P<0.05).

Table 3: *Average daily intake of milk replacer (MR) and nutrients. The MR intake show the mean values ± SEM. For the total intake, no SEM and P-values could be calculated because hay was offered to entire feeding groups. Means are different (P<0.05) between treatment groups. *** P<0.001, ** P<0.01; * P<0.05, # 0.05<P<0.1; NS = not significant. Control group (L0), 1% lactulose L1), 3% lactulose (L3).*

	Experimental group				*group differences (P-values)*		
Intake per day	**L0**	**L1**	**L3**	**Pooled SEM**	**L0 vs. L1**	**L0 vs. L3**	**L1 vs. L3**
MR intake:							
MR [g DM]	2080	2019	2199	45	NS	*	**
male	1958	1934	2133	73	NS	NS	NS
female	2201	2104	2264	26	*	NS	**
Energy [MJ ME]	35	34	37	1	NS	*	**
Crude protein [g]	455	452	514	10	NS	***	***
Ether extract [g]	410	398	433	9	NS	*	**
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 fiber [g]	68	69	70				
Ether extract [g]	409	398	437				

The higher daily MR intake of group L3 increased the average daily crude protein intake by about ~13 % compared to group L0. Higher average daily weight gain (ADG) was achieved by increasing the content of crude protein in MR [77]. The higher daily MR intake might be the result of the sweeter feed formulation by applying 3% lactulose regimen.

In total the ADG tended to be higher for L3 than L1 and was numerically higher for L3 than L0 (Tab. 4). Mainly in group L3 a positive trend on growth performance was determined, especially for male calves (P<0.1). A higher body weight was achieved during the 19 week treatment time and it can be asserted to be the effect of lactulose feeding [68]. The trends of the daily weight gain of female and male calves are presented in detail in figure 11.

Table 4: *Mortality and adjusted means ± SEM of body weight (BW) and feed efficiency of calves fed with MR containing E. faecium (L0) or added additional with lactulose (L1 and L3). Means are not different (P>0.05) between treatment groups. # P<0.1; NS = not significant. ADG = average daily gain; FE = feed efficiency, expressed as ADG [g/d] / MR intake [g/d].*

Variable	L0	L1	L3	Pooled SEM	L0 vs. L1	L0 vs. L3	L1 vs. L3
Calves [n]	14	14	14	0	NS	NS	NS
Mortality [n]	0	0	0	0	NS	NS	NS
Initial BW [kg]	74	74	74	2	NS	NS	NS
Final BW [kg]	244	245	255	6	NS	NS	NS
ADG: week 1-19 [g/d]	1288	1276	1350	59	NS	NS	#
FE	0.59	0.61	0.59	0.07	NS	NS	NS

Additionally, male calves generally show a higher and continuous increase in sex-hormone concentration in the pre-pubertal development than female calves. Thereby, the anabolic effect plays a major role in the weight gain and feed utilization. Several reports have shown that supplementing lactulose enhanced growth in pigs or calves [31,32,33]. Other researches about the effect of lactulose in farm animals observed no additional benefits [34]. This is understandable since the initial status of the microbial colonization of the intestine can differ widely between studies. 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 [78].

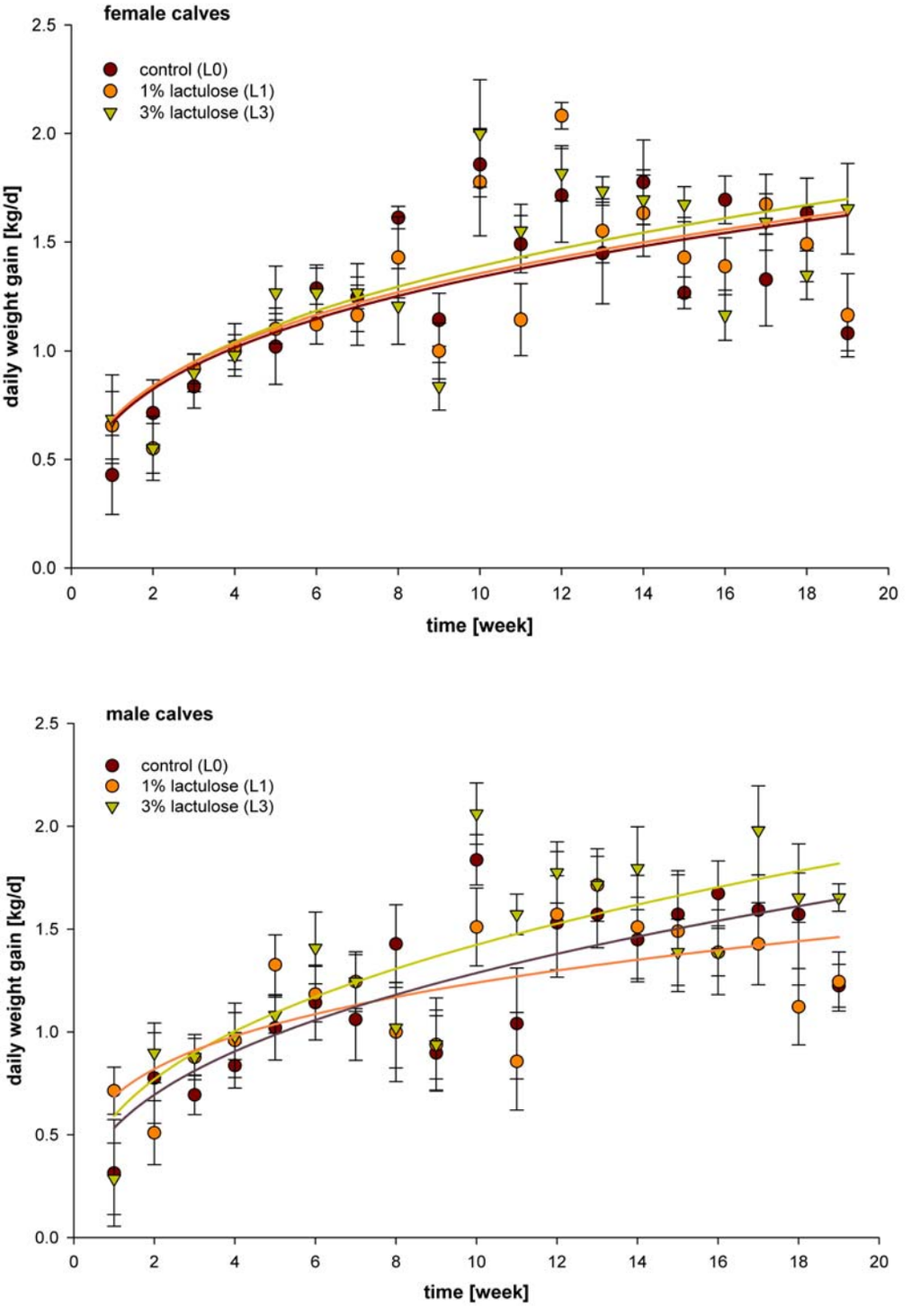


Figure 11: *Progression of the daily weight gain of male and female calves. The data were fit using the S-shaped function, which presents the trend of the daily weight gain.*

Although a higher live weight by the use of the probiotic *E. faecium* in all groups could be the reason for the non meaningful results in the development of growth performance. Higher life weight gains and lower feed inputs are achieved by the use of probiotics (Tab. 5). The control group could

achieve higher weight gains than a control group without feeding the probiotic bacteria. Thus, in the presented study there is a little scope for improved zootechnical performance.

Table 5: *Influence of probiotics on weight gain and feed conversion ratio in different branches of production (modified according to [45]).*

	Weight gain as % to the control group	***Feed conversion ratio in % to the control group***
Piglet breeding	Mean of 8 Studies: **+ 4.8** Range: (- 8.1 to + 24.3)	Mean of 8 studies: **- 1.5** Range: (+ 3.1 to - 9.3)
Calve breeding	Mean of 7 studies: **+ 5.4** Range: (- 5.3 to + 21.7)	Mean of 7 studies: **- 2.5** Range: (+ 3.6 to - 7.9)
Pig fattening	Mean of 9 studies: **+ 3.7** Range: (- 0.3 to + 6.7)	Mean of 9 studies: **- 5.1** Range: (- 1.4 to - 7.1)
Cattle fattening	Mean of 5 studies: **+ 4.8** Range: (- 4.3 to + 7.2)	Mean of 5 studies: **- 1.5** Range: (- 7.6 to - 4.7)

It has been suggested that probiotics are strain specific, species and dose specific. Also the synbiotic effects are different between used pre- and probiotic [46]. Consequently, the lactulose might not be the perfect substrate for *E. faecium* in the gut. An accurate and reliable prediction of the lactulose efficacy is therefore not possible.

Effect on the intestinal morphology

The influence of oral administered lactulose on the morphology of the intestine and on the expression of apoptosis and cell proliferation markers in the GIT was demonstrated in calves [68,79]. Based on histomorphological analyses, lactulose treatment was shown to decrease the villus height in ileum (P<0.001) of both treatment groups (Fig. 12). In contrast, Pelicano et al. (2005) [80] found no differences in the histomorphometrical parameters of the intestinal epithelium with the use of prebiotics in broiler chickens. Factors like species and growth phase of animals, type of dosing used, hygienic conditions of the housing and also the feeding matrix may explain variations in results of different studies. However, the decreased villus height in the ileum yields to a reduced resorption surface. In many cases the reduced surface correlates with an intestinal irritation. The positive effect of the smaller surface is the reduced aggregation of pathogens and toxins. The reason for the morphological differences could be a decreasing cell proliferation or/and an increasing apoptosis caused by the feeding of lactulose. Apoptosis is an important process responsible for maintenance of the cellular balance between proliferation and death and crucial for normal morphology and function in the GIT [81]. This form of cell death can be induced by a wide range of cellular signals, which leads to activation of cell death which is characterized by distinct

morphological changes [82]. Prebiotics are proven to increase apoptosis in the intestine [83] and especially lactulose reduced the cell proliferation [31]. Although a higher lactulose concentration in the ileum compared to the jejunum [84,85] might be a reason for the effect on villus heights only in the ileum.

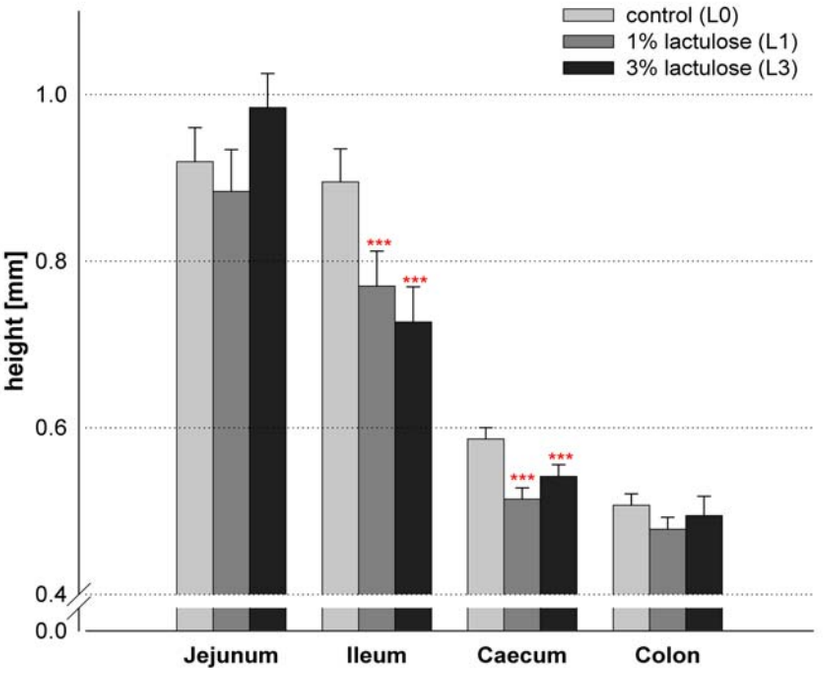


Figure 12: *Villus height and depth of crypts in the intestine. The villus heights are shown for jejunum and ileum. Crypts depth is shown for caecum and colon. Significant effects of lactulose are marked with an asterisk (*** P<0.001).*

The decreasing crypts 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 SCFA, like butyrate along with acetate and propionate, in the lumen of the hindgut by bacterial fermentation of lactulose was assigned in previous studies as reason for this morphological effect [86]. A number of different studies reported about lower colonic cell proliferation by increased synthesis of butyric acid [31,87]. In the large intestine this could possibly lead to a shortening of the crypts as it was presented in our study. However, lactulose yielded high proportions of acetic acid and low proportions of butyric acid [88,89]. For further assertion analysis of the butyric acid concentration in the chyme should be conducted in calves.

Changes in the intestinal mRNA gene expression of apoptotic markers were analyzed to confirm the histomorphometrical investigations. Our data demonstrate significant TGF-ß$_1$ (P<0.05) and Caspase 3 (P<0.05) mRNA increases in the caecum of the 3% lactulose treated calves, while diverse cell proliferation markers were not affected by the treatment. TGF-ß$_1$ is a multifunctional cytokine that regulates many diverse cellular processes including proliferation, apoptosis, differentiation [90] and immune regulation. It is the main cytokine in intestinal epithelial cells, which regulates epithelial differentiation and anticipate the damage of epithelia [91]. Caspase are the final

executioners of apoptosis, activated during a signaling cascade in almost all cell types [92]. Because apoptosis is important for the cellular balance in the intestinal morphology, an enhanced apoptotic rate seems to be associated with a decrease in crypt depth due to lactulose supplementation. Other morphological differences in the caecum could not be observed. Tumor necrosis factor alpha (TNF-α), a possible mediator for the induction of caspase activity and epithelial cell apoptosis in the GIT was also not found to be regulated at the mRNA level in this tissue. These cytokines, which are released in large quantities upon activation of intra-epithelial lymphocytes, have been shown to play a dynamic role in the homeostasis of the villus epithelial barrier [30,40,14]. Further on, the dominant anti-apoptotic marker Bcl-xl was down regulated in the jejunum by the oral application of 3% lactulose. It is known as the survival protein because of its cell death repressor activity and its interaction with the epidermal growth factor receptor (EGFR). A decreased anti-apoptotic rate seems to be associated with the tendency increase in villus heights in the jejunum [79].

In the GALT the PP are the main component and especially present in the ileum [93]. They are counted among the primary lymphoid organ and play a major role in the development of B cells [93]. 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. In the assessment the lymph follicle decreased significantly in the supplemented group ($P<0.01$), which is explained with the stabilization of the intestinal environment, by the reduction of pathogen bacteria which leads to a reduced activation of the immune system. Therefore 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.

Hematology

Changes in the number of thrombocytes and lymphocytes due to lactulose treatment could be determined. A significant higher number of lymphocytes in the L3 group ($P=0.02$) versus the control group and a lower number of thrombocytes in the treatment group L1 was detected (Fig. 13). Other hematology values were in a normal physiological range, according to Monke et al. [94].

Finally, SCFA production, particularly butyrate, in the colon may reduce the requirement of epithelial cells for glutamine, thereby sparing it for other cells, such as those of the immune system [95]. This hypothesis is supported by the observation that lactulose administration can increase serum glutamine levels [96], and glutamine is an essential energy source for lymphocytes [97]. Also a high protein intake could increase the number of lymphocytes, whereas it is unsettled if the effect is relative or absolute [98].

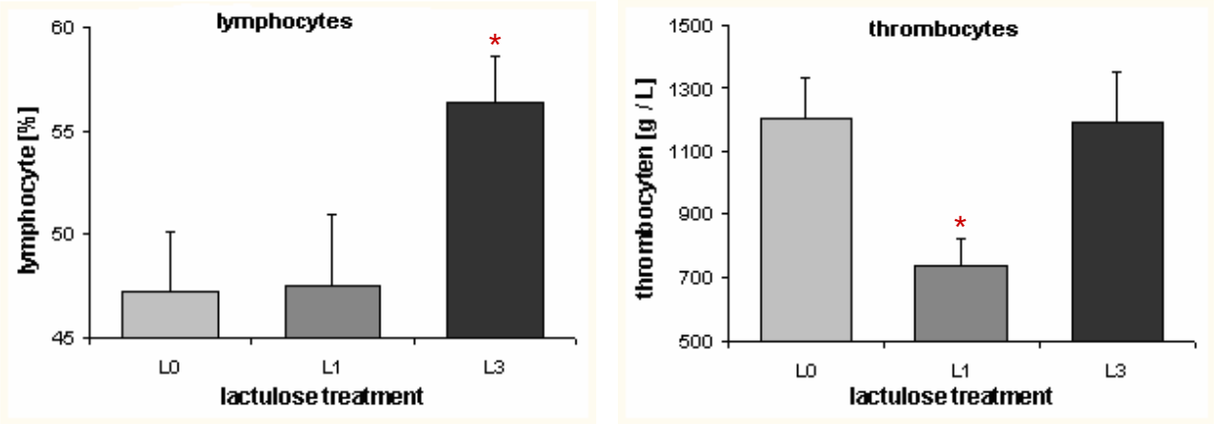
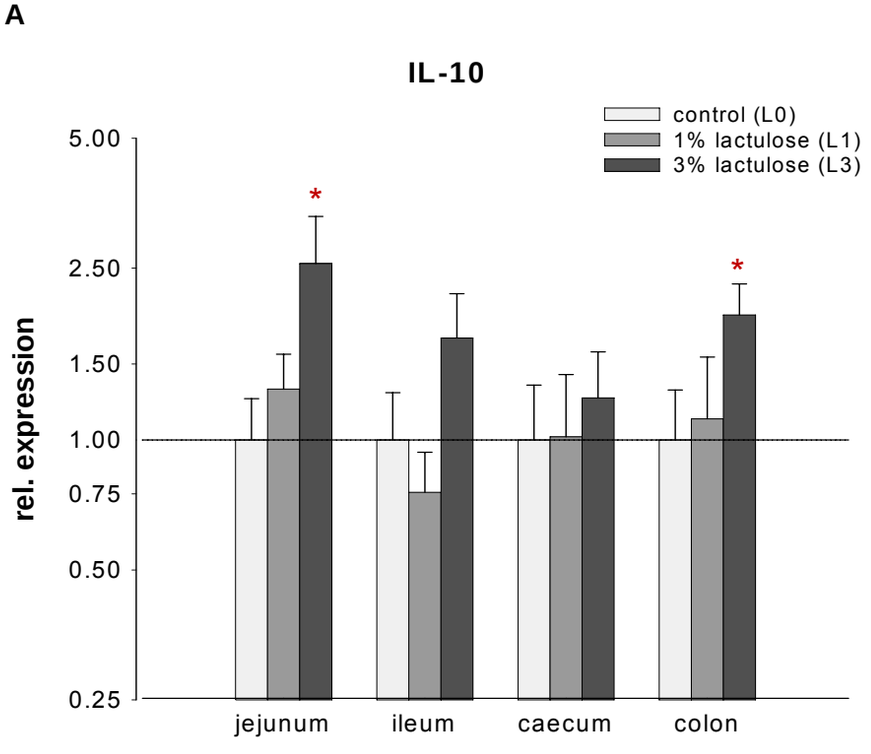


Figure 13: *Influence of lactulose feeding on lymphocytes and thrombocytes in probiotic feed calves.*

Immunological response in the intestine

Stimulation of the GALT via probiotics and prebiotics might influence immune modulation because of their ability to enhance production of IL-10 and TGF-β_1, both anti-inflammatory cytokines, which might have an essential role in subduing allergen-induced responses. Overall, the 3% lactulose feeding significantly stimulated IL-10 production in the jejunum and colon (Fig. 14).



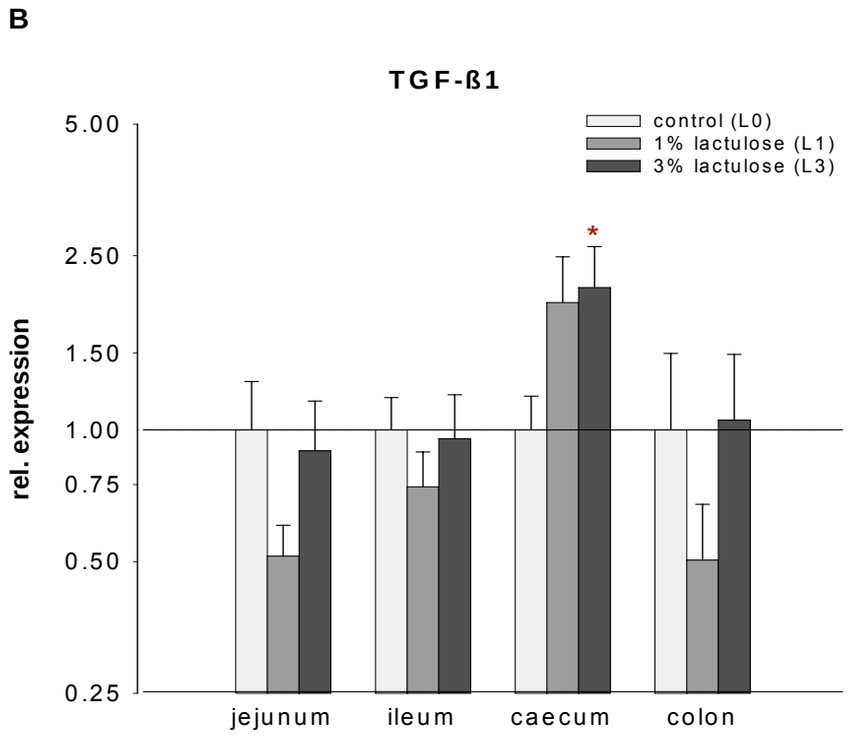


Figure 14: *IL-10 (A) and TGF-ß$_1$ (B) mRNA expression changes due to lactulose. Data are presented as relative expression in means ± SEM (n=14). Up-regulated genes are shown as values higher than 1 and down-regulated genes as values lower than 1. Significant effects of lactulose are marked with an asterisk (* P<0.05) and trends of regulation are shown with plus (# 0.05<P<0.1). The control groups have a constant level of 1 ± SEM.*

IL-10 is primarily produced by T helper 2 (Th2) and 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 [99]. Recent studies with mice also reported that consuming diverse prebiotics (fructo-OS or inulin enriched with oligofructose) enhanced IL-10 production [46,47]. Furthermore, the high dose lactulose treatment significantly stimulated TGF-ß$_1$ production in the caecum. TGF-ß$_1$ plays a decisive role in the development of the mucosa, due to the effect on B cells, which dispose a class switching to IgA.

Immune modulation in different compartments

By the analysis of gene expression differences between the feeding groups (male and female were analyzed together in one feeding group), the gene expression of all studied anti- and pro-inflammatory cytokines and diverse CD markers were not significant affected by lactulose feeding. On closer examination, varieties between the genders in the gene expression could be determined. The supplementation of 1% lactulose induced more significantly immunomodulation in female calves, whereas the supplementation of 3% lactulose affects the male calves in different immune compartments (Tab. 6).

Table 6: *Effect of lactulose on the mRNA expression of diverse cytokines, CD markers and IgA FcR in various calf tissues, compared to the untreated control group (L0). Significant down-regulations are marked with an arrow downwards (↓; P<0.05), (↓↓; P<0.01) and (↓↓↓; P<0.001). Up-regulations are marked with an arrow upwards (↑; P<0.05), (↑↑; P<0.01) and (↑↑↑; P<0.001) and trends of regulation are shown with #; 0.05<P<0.1. For no differences a blank was used.*

tissue	Treatment & sex		IL-10	TGF-ß$_1$	IFN-γ	TNF-α	IL-1ß	IL-8	IgA FcR	CD4$^+$	CD8$^+$	CD25	CD69
PP ileum	L1	male							↑				
		female								↓			
	L3	male	↓ (#)		↓				↑ (#)				
		female											
mLN	L1	male								↑ (#)			
		female								↓↓			
	L3	male										↓	
		female											
spleen	L1	male											
		female											
	L3	male											↓
		female											
WBC	L1	male											
		female					↑ (#)				↑		↑ (#)
	L3	male				↓ (#)							
		female											

The results demonstrate that the ileum and mLN are the primary tissues that specifically affected by lactulose feeding. Only the male calves of group L3 showed a down-regulation of IFN-γ about 67%. IFN-γ released by helper T1 (Th1) cells activate macrophages, induce B cells to switch immunoglobulin type [100] and it is important in regulating the humoral immunity (Th2 response). The reduced IFN-γ production of male calves by 3% lactulose treatment let assume that lactulose enhances the humoral immune response due to the fact that IFN-γ inhibited this response. Probiotic bacteria have been shown to enhance the humoral immune response and thereby promote the intestine immunologic barrier [61]. The synbiotic application of lactulose and *E. faecium* could amplify this effect. Substitutional for the Th2 response we used the IL-10 that is known to be immunosuppressive, capable of inhibiting the antigen presentation and the production of TNF-α and IL-1ß in activated monocytes/macrophages [101]. Male calves of group L3 showed a trend of down-regulation of 65% for IL-10 in the ileum. Other studies, using Inulin as prebiotic in combination *Lactobacillus rhamnosus* and *Bifidobacterium lactis* found an up-regulation of IL-10 [46]. It is still unclear whether probiotic bacteria and prebiotics modulate the activity of Th1/Th2 cells. The combined application of probiotics and prebiotics has different effects from those of the individual supplements, but does not simply result in additive or synergistic effects [46]. Different studies with mice or rats reported that consuming fructo-OS enhanced IFN-γ and IL-10 production by PP cells [46,47]. An synbiotic treatment abrogated the strong effects of prebiotics on PP cytokine

production [46,47]. Prebiotics and/or its metabolites may interfere specifically with the regulatory processes of cytokine production. The synbiotic treatment did not affect spleen or mLN, which constitutively express lower levels of multiple cytokine transcripts than PP in the ileum [102]. The low correlation between the level of production of IL-10 and IFN-γ (r=0.38) suggest that the lactulose treatment did not simultaneously activate different T-lymphocyte subpopulations and/or dendritic cells. A study with rats found that a probiotic feeding modestly affected immune functions, whereas systemic immunomodulatory effects were observed in rats fed synbiotics [46].

Subpopulations of T cells include the helper T cells, which are identified by the presence of the membrane glycoprotein $CD4^+$, and cytotoxic/suppressor T cells that express the $CD8^+$ glycoprotein [103]. $CD4^+$ cells secrete a number of cytokines that are important in the activation of B cells and other T cells, as well as cells of the innate immune system, whereas $CD8^+$ cells play a role in immunological tolerance, such as the tolerance to foreign antigens encountered in the gut [104].

Overall, the treatments did not affect the subpopulations of lymphocytes in male calves, whereas the $CD4^+$ T-lymphocytes of the 1% lactulose treated female calves decreased about 51% in the PP and about 60% in the mLN. In the same treatment group a 2.1-fold increase of the $CD8^+$ T-lymphocyte expression could be observed in WBC. Other studies conducted with acknowledged prebiotic fibers have shown an alteration in the proportion of T cells ($CD4^+$ and $CD8^+$) and increased lymphocyte and leukocyte numbers in the GALT of canine [105] and in the peripheral blood of calves [106]. Our results are conforming to these studies though lactulose increased the number of lymphocytes in blood (Fig. 13). A limited number of studies assessing the effect of lactulose or synbiotica on immune function have been published. Furthermore, results of several studies have been inconsistent and thus they were not directly comparable. Our results suggest that lactulose have only minor effects on the composition of T cell subsets in different immune compartments in calves.

The activation, differentiation, trafficking, and migration of T cells through sites of inflammation or infection are essential for an effective immune response. The α chain of the IL-2 receptor, CD25, is expressed on activated T cells, B cells and monocytes. Formation of the high-affinity IL-2 receptor allows T cell proliferation and differentiation to be driven by IL-2. The supplementation of 3% lactulose reduced the expression of CD25 mRNA in mLN (57%) and CD69 in spleen (65%) of male calves indicating that lactulose is able to modulate the activation status of *in vivo* differentiated T cells. Low expression of CD69, a marker of leukocyte activation, on $CD8^+$ cells could be attributed with a specific activation of CD3/$CD8^+$ T cells [107]. Only natural killer cells, which were activated by different nonpathogenic bacterial strains, are able to up-regulate activation markers [107].

Immunologic responses to pathogens are mediated by the IgA FcR. The IgA FcR is a trans-membrane glycoprotein present on the surface of myeloid lineage cells such as neutrophils, monocytes, macrophages and eosinophils [108]. Ligation of FcR by Ig-coated targets can trigger numerous cellular effector functions including phagocytosis, antibody-dependent cell-mediated cytotoxicity, respiratory burst, synthesis and release of cytokines and other inflammatory mediators [109]. In our study the treatment with lactulose increased the expression of the IgA FcR in male calves [69]. The availability of lactulose in the ileum may support growth of the supplemented probiotics and consequently stimulated IgA FcR synthesis. Additionally, studies have documented

that feeding lactulose is associated with increases in IgA secretion or IgA cells in GALT [110]. Thus, FcR provide a crucial link between the humoral and cellular arms of the immune system [111]. Upon activation, B cells undergo class-switching to produce IgA antibodies, a process that is facilitated by both activated $CD4^+$ and $CD8^+$ cells [112]. Furthermore, IFN-γ stimulates expression of the secretory component for IgA by epithelial cells [46]. Also, the high-affinity FcγRI is not constitutively expressed, a high level on neutrophils and cytokines such as INF-γ need to be administered to up-regulate receptor expression. However, no correlation between changes in IFN-γ production and IgA FcR expression in the ileum by lactulose treatment was found. It is a matter of common knowledge that prebiotics as well as probiotics increased the secretoric IgA in the GALT. In which way lactulose (or prebiotics in general) influenced the IgA FcR in still unclear.The results obtained are sometimes variable, but bearing in mind the different ways and conditions under which pre- and probiotics may be operating, it is not surprising that they are sometimes not active. Different strains of the same species may have different metabolic activities which affect the result when they are used as probiotics. Other factors which may also explain variation in results include the growth phase of the animal, the type of dosing used and the hygienic condition of the housing [36].

CONCLUSION

Our data suggest that RNA quality control prior to qRT-PCR assays is indispensable. Tissue sampling, RNA extraction and storage are very sensitive to RNA integrity and should be designed to keep RNA pure and intact. Total RNA samples of high quality (RIN>8) can serve as an optimal template whereas for partly degraded RNA (8>RIN>5) result in sub-optimal qRT-PCR expression results. Degraded RNA interferes with PCR performance as such, expressed as Ct value, whereas PCR efficiency is minor effected by RNA integrity. PCR efficiency seems to be major affected by the tissue type and extraction procedure. The delta-delta Ct and the efficiency corrected model are sensitive to RNA integrity. Statements about importance of normalization could be confirmed by our investigations, consequently we recommend an efficiency-corrected relative quantification strategy and normalization with an internally reference gene for every quantitative mRNA expression analysis. In view of the observed difference in gene expression stability between intact and degraded RNA sample, we and other authors propose performing RNA quality control prior to downstream quantification assays. We can recommend a RIN value higher than five and a PCR product length up to 200 bp as a minimal requirement for a successful and reliable real-time RT-PCR quantification.

A relatively small number of research trials were reported concerning supplementation of lactulose and other oligosaccharides in the diets of companion animals and livestock. Feeding MR containing *E. faecium* with 3% lactulose tends to result in increased feed intake and body weight gain. A 1% lactulose feeding shows no effect on growth performance. The effects of lactulose are obviously gender-specific: male calves tended to have an increasing body weight gain and female calves tended to have more changes in intestinal morphology in response to lactulose. This study indicates that lactulose feeding in combination with *E. faecium* affects the morphology of the small and large intestine in pre-ruminant calves. The reduced surface in intestine could lead to a decreasing aggregation of pathogens in the intestine. This assumption was confirmed by the reduced GALT activation via the PP in the ileum. Our data suggest that the use of lactulose could be meaningful when calves have intestinal irritation. By comparing expenses and gain of performance the economic profit in raising calves might be questionable. However, more research remains to be done to determine the appropriate role of lactulose in the nutrition of calves and to test the interaction between lactulose and probiotics such as *E. faecium*.

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SCIENTIFIC COMMUNICATIONS

Original publications

Fleige S and Pfaffl MW (2006):
RNA integrity and the effect on the real-time qRT-PCR performance.
Molecular Aspects of Medicine 27: 126-139.

Fleige S, Walf V, Huch S, Prgomet C, Sehm J, Pfaffl MW (2006):
Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR.
Biotechnology letters 28:1601-1613.

Fleige S, Preißinger W., Meyer HHD, Pfaffl MW (2007):
Effect of lactulose on growth performance and intestinal morphology of pre-ruminant calves using a milk replacer containing *Enterococcus faecium*.
Animal 1: 367-373.

Fleige S, Preißinger W., Meyer HHD, Pfaffl MW (2007):
Lactulose: Effect on apoptotic- and immunological markers in the gastrointestinal tract of pre-ruminant calves.
Veterinarni Medicina 52(10): 437-444.

Fleige S, Preißinger W., Meyer HHD, Pfaffl MW (2007):
The immune-modulating effect of lactulose in combination with *Enterococcus faecium* in pre-ruminant calves.
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Scientific presentations

Fleige S and Pfaffl MW:
Effect of RNA integrity on PCR results.
EMBO Practical Course on Quantification of gene expression by real-time qRT-PCR.
28.05-02.06.2005, EMBL, Heidelberg.

Fleige S, Preißinger W, Meyer, HHD, Pfaffl MW:
Einfluss einer synbiotischen Fütterung mit Lactulose und *Enterococcus faecium* auf die Darmgesundheit bei Milchkälbern.
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Posters

Meyer HHD, Fleige S, Prgomet C, Pfaffl MW:
Lactulose, Lactoferrin and Lactoferricin: Health benefits in the gastrointestinal tract.
27th IDF World Dairy Congress, 20-23.10.2006, Shanghai.

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Relative mRNA quantification models and the impact of RNA integrity.
3rd International qPCR Event, Technical University of Munich,
26-30.03.2007, Freising-Weihenstephan.

Fleige S, Meyer HHD, Pfaffl MW:
Lactulose: Health benefit in the gastrointentinal tract of probiotic fed calves.
Milchkonferenz 2007 der Deutschen Gesellschaft für Milchwissenschaft, 17-18.09.2007, Wien.

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APPENDIX

International reviewed publications of the author

Appendix I: Fleige S and Pfaffl MW (2006):
RNA integrity and the effect on the real-time qRT-PCR performance.
Molecular Aspects of Medicine 27: 126-139.

Appendix II: Fleige S, Walf V, Huch S, Prgomet C, Sehm J, Pfaffl MW (2006):
Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR.
Biotechnology letters 28:1601-1613.

Appendix III: Fleige S, Preißinger W., Meyer HHD, Pfaffl MW (2007):
Effect of lactulose on growth performance and intestinal morphology of pre-ruminant calves using a milk replacer containing *Enterococcus faecium*.
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Lactulose: Effect on apoptotic- and immunological markers in the gastrointestinal tract of pre-ruminant calves.
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Papers in preparation of the author

Appendix V: Fleige S, Preißinger W., Meyer HHD, Pfaffl MW (2007):
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Review

RNA integrity and the effect on the real-time qRT-PCR performance

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Abstract

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Working with low-quality RNA may strongly compromise the experimental results of downstream applications which are often labour-intensive, time-consuming, and highly expensive. Using intact RNA is a key element for the successful application of modern molecular biological methods, like qRT-PCR or micro-array analysis. To verify RNA quality nowadays commercially available automated capillary-electrophoresis systems are available which are on the way to become the standard in RNA quality assessment. Profiles generated yield information on RNA concentration, allow a visual inspection of RNA integrity, and generate approximated ratios between the mass of ribosomal sub-units. In this review, the importance of RNA quality for the qRT-PCR was analyzed by determining the RNA quality of different bovine tissues and cell culture. Independent analysis systems are described and compared (OD measurement, NanoDrop, Bioanalyzer 2100 and Experion). Advantage and disadvantages of RNA quantity and quality assessment are shown in performed applications of various tissues and cell cultures. Further the comparison and correlation between the total RNA integrity on PCR performance as well as on PCR efficiency is described. On the basis of the derived results we can argue that qRT-PCR performance is affected by the RNA integrity and PCR efficiency in general is not affected by the RNA integrity. We can recommend a RIN higher than five as

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good total RNA quality and higher than eight as perfect total RNA for downstream application.

Keywords: RNA quality; RNA quantity; qRT-PCR; 2100 Bioanalyzer; Experion; Lab-on-chip

Contents

1. The particular importance of RNA quality

1.1. Introduction

The accuracy of gene expression evaluation is recognised to be influenced by the quantity and quality of starting RNA. Purity and integrity of RNA are critical elements for the overall success of RNA-based analyses. Starting with low quality RNA may strongly compromise the results of downstream applications which are often labour-intensive, time-consuming and highly expensive (Raeymarkers, 1993; Imbeaud et al., 2005). It is preferable to use high-quality intact RNA as a starting point in molecular biological as well as in diagnostic applications. Especially in quantitative RT-PCR, micro-arrays, ribonuclease-protection-assay, in situ hybridization, northern blot analysis, RNA mapping, in vitro translation, cDNA library construction and any kind of array applications the integrity of the used total RNA should be checked. Especially in clinical application with unique or limited tissue material, e.g. obtained after surgery, a reliable RNA quality analysis is necessary (Bustin and Nolan, 2004b). As a consequence, several steps during tissue handling have to be carefully controlled in order to preserve the quality and integrity of the RNA material. It is well known that RNA is sensitive to degradation by postmortem processes and inadequate sample handling or storage (Perez-Novo et al., 2005). Thus the competency to quickly assess RNA quality using minor amounts has become increasingly important as the following measures of mRNA transcripts have become more expensive and more comprehensive.

1.2. RNA extraction

The quality and quality of purified RNA is variable and after the extraction during long storage rather unstable (Bustin et al., 2005). Especially long mRNA fragments up to 10 kb are very sensitive to degradation. This can happen through cleavage of RNAses introduced by handling with RNA samples. The most obvious problem concerns the degradation of the RNA and this is best addressed by insisting that every RNA preparation is rigorously assessed for quality and quantity. The extraction and purification procedure of total RNA must fulfill the following criteria (Bustin and Nolan, 2004b; Pfaffl, 2005a):

- free of protein (absorbance 260 nm/280 nm);
- free of genomic DNA;
- should be undegraded (28S:18S ratio should be roughly between 1.8 and 2.0, with low amount of short fragments);
- free of enzymatic inhibitors for RT and PCR reaction, which is strongly dependent on the purification and clean-up methods;
- free of any substances which complex essential reaction co-factors, like Mg^{2+} or Mn^{2+};
- free of nucleases for extended storage;

There are a substantial quantity of problems that affect reproducibility, and hence the relevance of results. The source of RNA, sampling techniques (biopsy material, single cell sampling, laser micro-dissection) as well as RNA isolation techniques (either total RNA or poly-adenylated RNA isolation techniques) often vary significantly between processing laboratories (Bustin and Nolan, 2004b; Pfaffl, 2004). The RNA quality can be different between two extraction methods, e.g. performed by hand or by an automatic extraction system. The isolated total cellular RNA with the liquid extraction, e.g. Trizol (Roche Diagnostics, Germany) or TriFast (peqlab, Germany), has different RNA quality, whereas only the type of homogenization is changed (Fleige and Pfaffl, 2006). Due to its inherent susceptibility to ubiquitous RNases and its chemical instability, RNA is readily endangered by base- or enzyme-catalyzed hydrolysis. Researchers must take into account a variety of factors, which influence their ability to obtain high-quality RNA that is free of contamination such as RNases, proteins and genomic DNA. These factors include yield variations, processing requirements, and sample availability of different cells or tissues. The best RNA yield is obtained from tissue that has been diced into small fragments with a scalpel prior to being frozen by submerging in liquid nitrogen. The samples must be homogenized using a bead mill or a mechanical homogenizer (Bustin and Nolan, 2004b).

Further problem may arise in the case of research on human or animal tissue sampling techniques and the time dependency until the tissue is stored safely in RNase inhibitors or RNA-later (Ambion, USA). It is often very challenging to decrease this sampling time to a minimum within the framework of clinical routine procedures, or in animal experiments during a slaughtering process. The RNA quality may also be

impaired in samples stored for a long time or under sub-optimal conditions (Schoor et al., 2003).

1.3. RNA quantity and quality assessment

Conventional methods are often not sensitive enough, not specific for single-stranded RNA, and disposed to interferences from contaminants present in the sample (Imbeaud et al., 2005). The assessment of RNA integrity can do by various methods: the classical gel OD measurement, modern OD measurement via NanoDrop, old fashioned denaturating agarose gel-electrophoresis or with high innovative lab-on-chip technologies like Bioanalyzer 2100 (Agilent Technologies, USA) and Experion (Bio-Rad Laboratories, USA). Quantity and quality assessment using a UV/VIS spectrophotometer should be performed at multiple wave lengths at 240 nm (background absorption and possible contaminations), 260 nm (specific for nucleic acids), 280 nm (specific for proteins), and 320 nm (background absorption and possible contaminations). On basis of the OD 260 the quantity, and by the ratio of the optical density (OD) of OD 260/280 the quality, OD 260/240 or OD 260/320 the purity and the extraction performance can be verified. An OD 260/280 ratio greater than 1.8 is usually considered an acceptable indicator of good RNA quality (Sambrook et al., 1989; Manchester, 1996). By the presence of genomic DNA the OD 260 measurement can compromised and leading to over-estimation of the actual and real RNA concentration. Further the used buffer and high salt concentrations will interfere with the result of the optical measurement and therefore the calculated RNA concentrations might be over- or under-estimated (own unpublished results). The accuracy of the OD 260/A280 method has been questioned, with a value of 1.8 corresponding to only 40% RNA, with the remainder accounted for by protein (Bustin and Nolan, 2004b).

More modern spectrometric methods, like the NanoDrop (ND-3300, NanoDrop Technologies, USA) in combination with RNA RiboGreen dye (Molecular Probes, Invitrogen, USA) can be used as an UV/VIS spectrophotometer for ultra sensitive quantification of RNA. A major advantage of the system is the very low sample consumption of 1–2 μl, which is especially important when using precious materials like human biopsy or laser dissected samples. Since the sample is not contained in a secondary vessel, the sample directly wets the system optics, reducing the variations and contaminations resulting from changing or repositioning the cuvettes. Further the ND-3300 measure a spectra of your sample covering 400–750 nm, giving you more information about the RNA integrity and other chemical contamination or the extracted RNA (ND-3300 user manual V2.5, NanoDrop Technologies, USA).

An additional check involves gel electrophoresis with RNA either stained with SYBR Green dye (Molecular Probes) or the less sensitive ethidium bromide (Bustin and Nolan, 2004b). But the assessment of RNA integrity by inspection of the 18S and 28S ribosomal RNA bands using denaturating gel electrophoresis is a cumbersome, low-throughput method and requires significant amounts of precious RNA (Bustin and Nolan, 2004a). Using the RiboGreen (Molecular Probes) reagent, the detection as little as 1 ng RNA/ml is possible, and can be measured reproducible.

In contrast to UV absorbance measurements at 260 nm, where proteins and free ribonucleotides in the mixture interfere with accurate quantitation, the RiboGreen reagent only measures polymeric nucleic acids (Jones et al., 1998; LePecq and Paoletti, 1966; Karsten and Wollenberger, 1977).

Today high innovative lab-on-chip technologies like micro-fluidic capillary electrophoresis were used to do RNA quality and quantity assessments. Certainly, in terms of routinely analyzing large numbers of RNA preparations, it is by far the most convenient and objective way of assessing the quality of RNA. This method has become widely used, particularly in the gene expression profiling platforms (Bustin, 2002; Lightfood, 2002; Mueller et al., 2000).

The Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the Experion (Bio-Rad Laboratories, USA) provide a framework for the standardization of RNA quality control. Therefore RNA samples are electrophoretical separated on a micro-fabricated chip and subsequently detected via laser induced fluorescence detection. It requires only a very small amount of RNA sample down to 200 pg total RNA. The use of a RNA ladder as a mass and size standard during electrophoresis allows the estimation of the RNA band sizes. Integrity of the RNA may be assessed by visualization of the 18S and 28S ribosomal RNA bands. An elevated threshold baseline and a decreased 28S:18S ratio, both are indicative of degradation (Mueller et al., 2004). The intact RNA preparation (Fig. 1) shows high 18S and 28S rRNA peaks as well as a small amount of 5S RNA. Degradation of the RNA sample

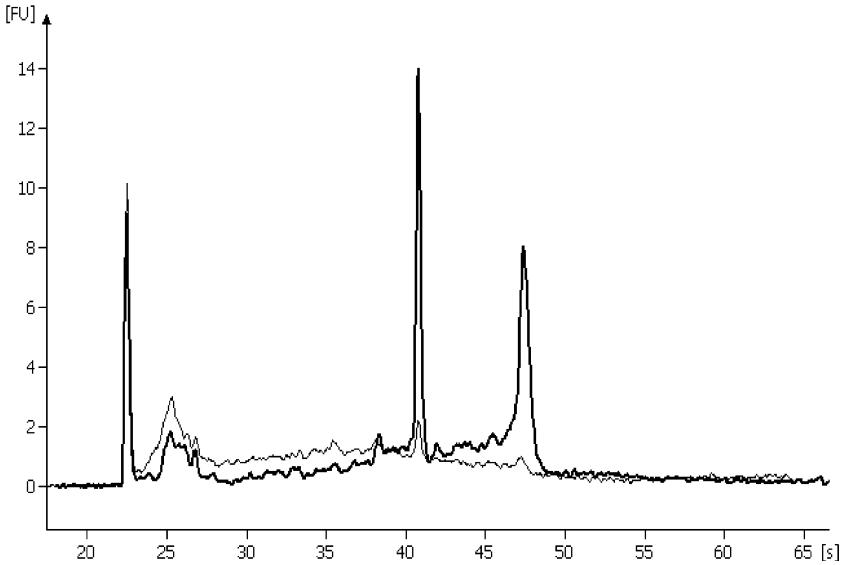

Fig. 1. Chromatograms of micro-capillary electrophoresis from RNA samples showing different degrees of degradation. A typical electropherogram of high-quality RNA (*solid black line*, RIN = 7.5) include a clearly visible 28/18S rRNA peak ratio and a small 5S RNA. Partially degraded sample (*thin grey line*; RIN = 4.5) was indicated by a shift in the electropherogram to shorter fragment sizes and produce a decrease in fluorescence signal as dye intercalation sites are destroyed.

produces a shift in the RNA size distribution toward smaller fragments and a decrease in fluorescence signal as dye intercalation sites are destroyed. The 28S/18S ratios are automatically generated by the both software applications in Experion and Bioanalyzer 2100. The RNA measurement using the lab-on-chip technology appears stable and relatively uninfluenced by contamination. RNA from tissue samples are typically classified according to the observation that the 28S rRNA peak area should be approximately twice the quantity of that of the 18S in total RNA samples for the mRNA quality to be acceptable (Sambrook and Russel, 2001). In general a 2.0 ribosomal ratio is regarded as perfect (Sambrook and Russel, 2001; Mueller et al., 2004). But in practice this value hardly is obtained. The 28S/18S ratio may reflect unspecific damage to the RNA, including sample mishandling, postmortem degradation, massive apoptosis or necrosis, but it can reflect specific regulatory processes or external factors within the living cells. As it is apparent from a review of the literature, the standard 28S/18S rRNA ratio of a 2.0 is difficult to meet, especially for RNA derived from clinical samples, and it now appears that the relationship between the rRNA electropherogram profile and mRNA integrity is up to now unclear (Monstein et al., 1995).

Furthermore, the generated ribosomal ratios are dependent on the used capillary-electrophoresis. In an intern study comparing Bioanalyzer 2100 (Agilent Technologies) with Experion (Bio-Rad) both capillary-electrophoreses systems showed differences in the generated ratio value, sensitivity, variation, and reproducibility (data not shown). Nevertheless, both platform showed more or less the same results.

However, it is unable to locate the original data for this commonly accepted premise. Based on structural differences alone, it might be expected that the in situ stability of mRNA differs from rRNA. Certainly, RNases will eventually result in the loss of both components, although there are other factors under which in situ rRNA will be completely degraded but mRNA remains intact (Mayne et al., 1999). Santiago et al. (1986) described that the mRNA integrity correspond more closely to the 28S than to the 18S integrity. This would mean that with increased length, there is a greater statistical chance of cleavage. Contrary to this assumption, Miller et al. (2004) expected that the 18S integrity correlated better than 28S with the mRNA, as the length of 18S is more closely aligned with that of the average mRNA. From our findings we can confirm the mRNA quality is more related to the 28S rRNA, which is often much faster degraded than the 18S. In a time dependent total RNA degradation via UV light the 28S rRNA disappeared very quickly (data not shown). Therefore the 28S/18S ratio has to be assessed for every single experiment and this is regarded as inadequate for the assessment of the quality (Marx, 2004). Altogether, it appears that the total RNA with lower rRNA ratios is not necessarily of poor quality especially if no degradation products can observe in the electrophoretic trace (Imbeaud et al., 2005).

A new tool for RNA quality assessment is the RNA Integrity Number (RIN, developed by Agilent Technologies) for the lab-on-chip capillary gel-electrophoresis used in the Bioanalyzer 2100 (Mueller et al., 2004). This tool is based on a neuronal network which determines the RIN number from the shape of the curve in the

electropherogram (Fig. 1). The software and the algorithm allows the classification of total RNA on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments. The verification of the RNA integrity before use in different applications permits to compare experiments and classify the significance of results (Mueller et al., 2004; Imbeaud et al., 2005).

The dependence of the RNA integrity on various calf tissue samples, white blood cells and four cell lines was determined (Fleige and Pfaffl, 2006). As shown (Table 1) for solid tissues the average RIN is between 6 and 8. Tissues or organs with high content of connecting tissue, e.g. in the gastrointestinal tract like rumen, omasum and jejunum, underlie high RNA degradation through the sampling and extraction procedure and show great RIN variations. The reason for this variability might be the solid and tough structure of the tissues, e.g. connecting or fatty tissue, the RNase enzymatic activity and problems during tissue sampling and storage. Furthermore tissues from the gastrointestinal tract have been washed in physical saline solution to get rid of any disturbing gut substances. Thus the physiological constitution of the tissue, the time and management of tissue sampling has a bearing on the degradation level of RNA. In contrary single cells like white blood cells (WBC) or cell derived from cell lines have higher RIN. Cell sampling and RNA extraction is much faster and easier, because cells are better accessible and were not kept in any suboptimal conditions. Therefore RNA integrity based on the RIN classification is

Table 1
Average RNA integrity numbers (RIN) of various bovine tissues and cell lines analyzes with the bioanalyzer 2100 (Agilent Technologies)

Tissue	Quality metrics		
	Mean	Std. dev.	*n*
Liver	6.49	±0.86	28
Heart	6.03	±1.19	23
Spleen	7.28	±0.60	17
Lung	6.55	±0.67	22
Rumen	**4.70**	**±2.81**	23
Reticulum	5.47	±1.29	21
Omasum	6.64	±1.87	18
Abomasum	7.30	±0.86	17
Ileum	7.35	±1.53	17
Jejunum	**4.56**	**±2.13**	20
Colon	7.52	±0.62	19
Caecum	7.28	±0.86	16
Lymph node	6.93	±0.65	26
Kidney cell	8.87	±0.32	3
Corpus luteum	9.62	±0.32	5
Granulosa cell	8.38	±0.41	5
Oviduct	9.40	±0.29	5
WBC	9.36	±0.13	5

much better and lay around RIN 9. The importance of isolation technique for a good RNA quality is shown in detail in Fleige and Pfaffl (2006).

2. Integrity of RNA and its effect on real-time qRT-PCR

2.1. General aspects

For a sensitive and reliable quantitative measurement of low abundant mRNA gene expression real-time quantitative reverse-transcription polymerase-chain-reaction (qRT-PCR) reaction is the method of choice. qRT-PCR shows high sensitivity, good reproducibility and a wide quantification range (Bar et al., 2003; Wang and Brown, 1999). For successful qRT-PCR and micro-array experiments it is important to use intact RNA. It is not known how this image is influenced by sample preparation factors which such as RNA quality, cDNA synthesis and labeling efficiency. Therefore the determination of RNA quality is a critical first step in obtaining meaningful data of gene expression.

Many factors present in samples as well as exogenous contaminants have been shown to inhibit the RT as well as the PCR. Some of them derive from the extracted tissue, others stem from inefficient or messy lab management. For example, the presence of haemoglobin, fat, glycogen, cell constituents, Ca^{2+}, high genomic DNA concentration, and DNA binding proteins are important factors (Wilson, 1997; Rossen et al., 1992). Exogenous contaminants such as glove powder and phenolic compounds from the extraction process or the plastic ware can have an inhibitory effect. Also unknown tissue-specific factors can influence amplification kinetics but this affect can be ameliorated, in part, by appropriate primer selection (Wilson, 1997; Rossen et al., 1992; Tichopad et al., 2004). There nevertheless, little is known about the possibility of obtaining reasonable qRT-PCR data from RNA samples with impaired quality. Expression differences for some genes can independently confirmed by real-time qRT-PCR. Gene Expression profiles obtained from partially degraded RNA samples with still visible ribosomal bands exhibit a high degree of similarity compared to intact samples and that RNA samples of sub-optimal quality. This might therefore still lead to meaningful results if used carefully (Schoor et al., 2003).

2.2. Effect on the mRNA quantification

In view of the observed difference in gene expression stability between intact and degraded RNA samples from the same tissue and the higher gene-specific variation in degraded samples, we propose performing RNA quality control prior to downstream quantification assays (Bustin and Nolan, 2004a). Especially if one aims to accurately quantify small expression differences (Perez-Novo et al., 2005). With that prospect in mind, and with the aim of anticipating future standards by pre-normative research, it is connotatively too identified and analyzed the influence of degraded RNA on the performance on qRT-PCR. In a study from Fleige and Pfaffl (2006) the purity and integrity of RNA samples was assessed, derived from different bovine

tissues and cell lines, using the Bioanalyzer 2100 (Agilent Technologies). To test the influence of the RNA integrity (numbered according to the RIN classification), the intact transcriptome of one distinct bovine tissue was degraded factitiously by enzymatic digest or with ultraviolet light. This leads to enzymatic cutoffs or breaks in the native RNA strand resulting in fragments of different lengths. A gradient with several steps of intact RNA (RIN 8–10) down to degraded RNA (RIN = 1–3) was investigated. The effect of RIN on qRT-PCR performance was investigated by correlating the RIN values with the crossing points (CP) of the PCR runs. The expression levels of four genes were assessed, all of different abundance levels ranging from high abundant 18S and 28S rRNA, intermediate abundant β-actin, down to very low expressed IL-1β mRNA samples. The importance of using high-quality RNA is demonstrated by the results shown in Fig. 2. A high-quality RNA (high RIN) determined a lower CP than by a less-quality (lower RIN). High significant relation between RIN and CP ($p < 0.01$ for the trend) could be shown for all examined genes ($n = 4$) and tissues ($n = 14$). With increasing RNA quality the variability of the qRT-PCR result was decreased (Huch et al., 2005).

It is well known, that normalization by an internal reference gene reduce or even diminish tissue derived effects on qRT-PCR (Wittwer et al., 1997). Reliability of any relative RT-PCR experiment can be improved by including an invariant endogenous control in the assay to correct for sample-to-sample variations in RT-PCR efficiency and errors in sample quantification. So called relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and gives a result relative to the levels of an internal control RNA (Pfaffl, 2001). For many

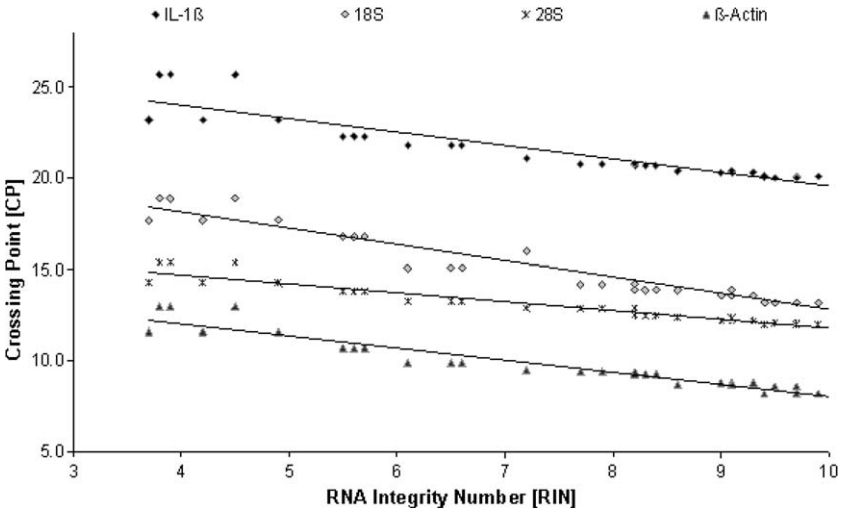

Fig. 2. Influence of RNA integrity (RIN) on the Crossing Point (CP): Amplification curves from three HKG (18S, 28S, β-Actin) and IL-1β with different quality of employed RNA from corpus luteum. An increase of RNA degradation correlates significantly to the amplified product, such that the CP is decrease with increasing RNA integrity number (RIN). Quantitative analyses use the threshold cycle number (Ct), at which the signal is detected above the background and is in the exponential phase.

experiments this method is most adequate for investigating physiological changes in gene expression levels. It is based on the expression levels of a target gene versus an internal reference gene, often non-regulated housekeeping gene are prominent candidates. To get rid of the RIN dependency the CP data were normalized by an internally expressed reference gene (Fleige and Pfaffl, 2006), according to the ΔCP method described earlier (Livak and Schmittgen, 2001). The normalized results (Fig. 3), expressed as RIN compared to ΔCP values showed minor influence of RNA quality on the expression results, and the significant effects could be reduced to a minimum.

Sometimes, even intact RNA does not guarantee good results because RNA sample may contain inhibitors that can reduce reaction efficiency (Bustin and Nolan, 2004a; Wong and Medrano, 2005). These factors include length of the amplicon, secondary structure and primer quality. The shapes of amplification curves differ in the steepness of any fluorescence increase and in the absolute fluorescence levels at plateau depending on background fluorescence levels. Therefore PCR efficiency has a major impact on the fluorescence history and the accuracy of the calculated expression result and in critically influenced by PCR reaction components. Efficiency evaluation is an essential marker in real-time gene quantification procedure (Tichopad et al., 2003, 2004). The effect of RIN on PCR efficiency was investigated similarly to the above mentioned tissues and various RNA qualities. The efficiency of all investigated genes was not affected by the RNA quality, independent of gene or tissue. A causally determined correlation between the RIN and the CP is shown in Fig. 4 (Fleige and Pfaffl, 2006).

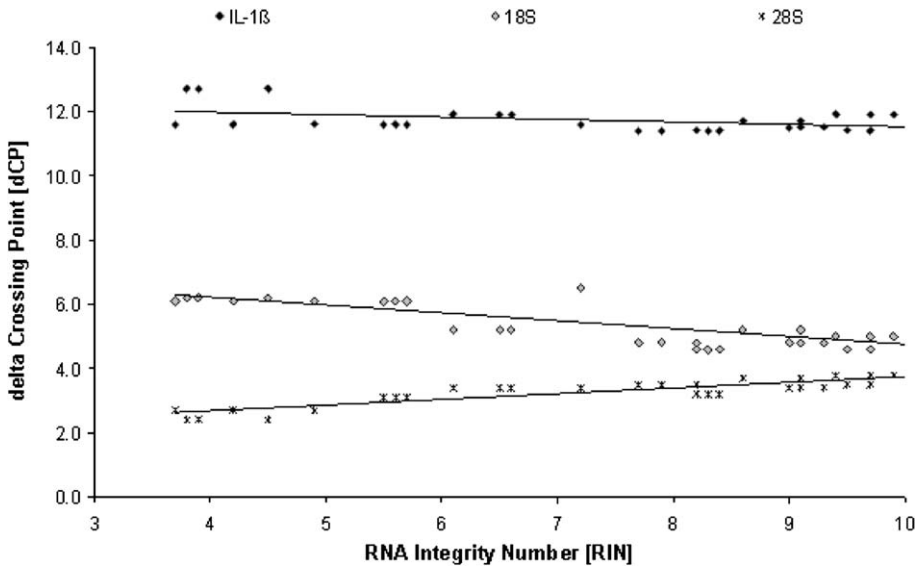

Fig. 3. Influence of RNA integrity (RIN) on the delta CP. The results (CP) from Fig. 2 are normalized with β-Actin. The significant effect of RNA integrity could reduce to a minimum.

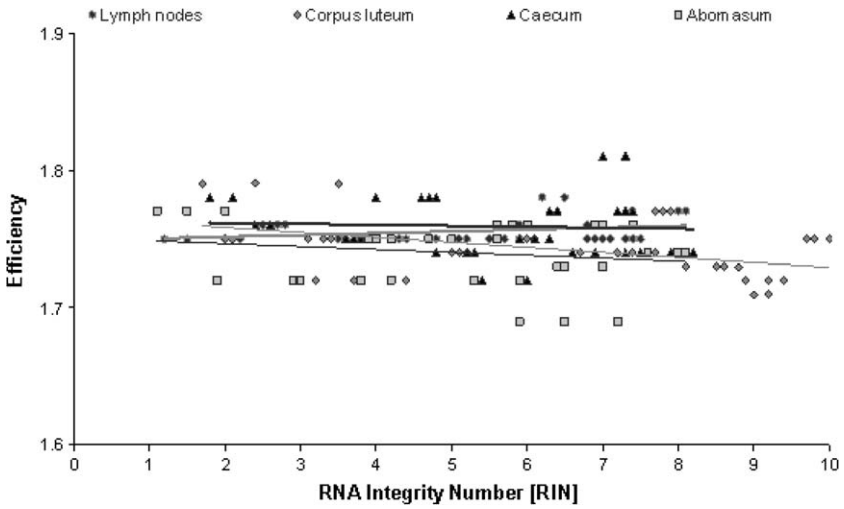

Fig. 4. Influence of RNA integrity (RIN) on PCR Efficiency: The Efficiency was generated by Rotor-Gene 3000 software (Corbett-Research). Only four tissues (lymphnode, corpus luteum, caecum, abomasums) were graph, additional results show the same trend.

2.3. Association between RNA quality and length of the amplified product

The PCR efficiency is also influenced by various factors, among other things by the annealing temperature, the primer length or by the length of the amplified product. And because of exponential amplification of the initial information, any extant error is amplified, too (Tichopad et al., 2002). The new question is, if the PCR efficiency during real-time qRT-PCR is influenced by the RNA quality or not? Therefore again, a gradient with several steps of intact RNA down to degraded RNA were examined with different primer sets, amplifying qRT-PCR products of various lengths. Primer sets for varying lengths of product (50–950 bases) were used to amplify the sequence of β-actin in different tissues and RNA integrity levels. The correlation between RNA integrity and CP were examined. The results of the correlation between the RIN and CP fulfilled the expectations. It is clearly visible that the crossing point is shifted towards lower cycle numbers using intact total RNA or higher RIN. With increasing length of the amplified product, the importance of RNA quality rises. Regarding the results concerning the correlation between the RIN and the CP values, there were some differences in the tested tissues. In some tissues a correlation between the RIN and the crossing point was visible for shorter products and in WBC and corpus luteum this correlation was visible as well for longer products.

In general we can point out, that amplification of long product over 400 bp is strongly dependent on a good RNA quality, which should show at least a RIN of 5. Shorter qRT-PCR products, mostly used with the length of 70–250 bp, are more or less "*independent*" of the RNA quality. Viewing the correlation between the RIN

and the efficiency of PCR, it is noticeable that the efficiency does not vary within one amplicon length, despite some exceptions. No correlation between the RIN and PCR efficiency (ranging between 1.6 and 1.7) was given (Pfaffl, 2005b).

Other studies showed as well an inhibitory effect of poor RNA quality on real-time PCR results. Degraded or impure RNA can limit the efficiency of the RT reaction and reduce yield. RNA should either be prepared from fresh tissue, or from tissue treated with an RNA stabilization solution such as RNA later (Labourier, 2003, 2004). The importance of using full length RNA for reverse transcription depends on the application. As a result, some degradation of the RNA can be tolerated. If it is not possible to use completely intact RNA, a design of primers to anneal an internal region of the gene of interest is useful. Note that for truly quantitative RT-PCR, partially degraded RNA may not give an accurate representation of gene expression (Wang, 2005).

3. Conclusion

In conclusion, while all efforts should be made to obtain high-quality RNA samples that reflect the natural state most reliably, moderately degraded samples with a degradation signature may still lead to a reasonable qRT-PCR expression profile. The normalized expression differences measured with the real-time RT-qPCR are similar to those obtained from high-quality samples. Only the non-normalized values show a correlation between RNA integrity and CP. This findings show the importance of the normalization. The reliability of any relative RT-PCR experiment can be improved by including an invariant endogenous control in the assay to correct for sample-to-sample variations in RT-PCR efficiency and errors in sample quantification. Furthermore, RNA samples of optimal quality can serve as a template for all product lengths whereas for degraded RNA primer pairs for shorter amplicon are more suitable. To be on the safe side with primer pairs it would be helpful to prove the RNA quality before starting the run.

Up to now it is still questionable if we can use the 28S/18S ratio or the RIN, both based on the quantity and quality check of the ribosomal sub-units, to make a definite statement on the mRNA quality which is our target in qRT-PCR. We are looking forward for sensitive methods, comparable to an intelligent algorithm, which prove the real mRNA integrity to have a reliable answer on mRNA quantity and quality.

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Appendix II:

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ORIGINAL PAPER

Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR

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Abstract Relative quantification in quantitative real-time RT-PCR is increasingly used to quantify gene expression changes. In general, two different relative mRNA quantification models exist: the delta-delta Ct and the efficiency-corrected Ct model. Both models have their advantages and disadvantages in terms of simplification on the one hand and efficiency correction on the other. The particular problem of RNA integrity and its effect on relative quantification in qRT-PCR performance was tested in different bovine tissues and cell lines ($n = 11$). Therefore different artificial and standardized RNA degradation levels were used. Currently fully automated capillary electrophoresis systems have become the new standard in RNA quality assessment. RNA quality was rated according the RNA integrity number (RIN). Furthermore, the effect of different length of amplified products and RNA integrity on expression analyses was investigated. We found significant impact of RNA integrity on relative expression results, mainly on cycle threshold (Ct) values and a minor effect on PCR efficiency. To minimize the interference of RNA integrity on relative quantification models, we can recommend to normalize gene expression by an internal reference gene and to perform an efficiency correction. Results demonstrate that innovative new quantification methods and normalization models can improve future mRNA quantification.

Keywords Gene expression study · Ct · mRNA · qRT-PCR · real-time RT-PCR · RNA integrity · RNA integrity number (RIN)

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Introduction

PCR for gene expression profiling has become the standard technology for the quantification of nucleic acids. For an exact quantitative measurement of low abundant mRNA gene expression real-time quantitative reverse-transcription PCR (qRT-PCR) is the method of choice. Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. This control RNA is often a classical reference gene, like GAPDH, ribosomal RNA subunits (18S and 28S rRNA), or β-actin, which are co-amplified in the same tube in a multiplex-assay or amplified in a

separate tube as an external standard (Bustin and Nolan 2004). The relative quantification strategy is adequate for most purposes to investigate physiological changes in gene expression levels.

An essential requirement for a successful quantitative mRNA analysis using qRT-PCR is the usage of intact RNA. Low-quality RNA may compromise the derived expression results. Investigations with low quality RNA as starting material supply results of qRT-PCR (Imbeaud et al. 2005; Raeymarkers 1993). The need to isolate high quality total RNA from wide variety of clinical and/or experimental tissue samples becomes more important for quantitative gene expression studies. After extraction the RNA is unstable. Long mRNA, up to several kilo bases, is very sensitive to degradation (Bustin 2002). This can occur through cleavage by RNAses during handling of RNA samples, otherwise it may also be impaired in samples stored for a long time or under sub-optimal conditions (Schoor et al. 2003). Consequently, the determination and confirmation of RNA quantity and quality is the first critical step in obtaining meaningful gene expression data.

Verification of RNA integrity prior to usage in downstream qRT-PCR application permits experiments to be compared and provide more accurate and reliable results. While methods for the physical isolation of total RNA have evolved significantly over the last two decades, there has been limited advancement in methods used for assessing RNA quality. Today it is well acknowledged that the accuracy of gene expression is influenced by starting RNA quality. RNA purity is normally assessed by its A260/A280 ratio (Baelde et al. 2001). The spectrometric methods often fail in sensitivity, are highly variable and give no results in terms of RNA integrity. In the past, RNA quality could often not be assessed exactly. The further development of the capillary gel electrophoresis methods and spectrophotometer technologies have addressed this issue (Auer et al. 2003).

The micro-fluidic capillary electrophoresis has developed into commonly tool, particularly in the gene expression profiling platforms (Mueller et al. 2000; Livak and Schmittgen 2001). Instruments, such as the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the Experion (Bio-Rad Laboratories, Hercules, CA), are becoming more and more standard since their use dramatically decreased the amount of RNA needed to evaluate integrity down to the submicrogram scale. On condition that the 18S and 28S ribosomal RNA (rRNA) fragments produce well-defined peaks without any smearing towards a smaller size, the sample can be considered for further analysis. The 28S/18S ratio is automatically generated and is the first criterion for a total RNA quality check. A new and more advanced tool for RNA quality assessment is the RNA Integrity Number (RIN, Agilent Technologies). The algorithm assigns a RIN number score from 1 to 10, where level 10 represents a completely intact RNA, and 1 presents a highly degraded RNA (Mueller et al. 2000). An interpretation of an RNA integrity is facilitated, comparison of samples is possible and repeatability of experiments is ensured.

While it is obvious that intact RNA constitutes the best representation of the natural state of the transcriptome, there are situations in which gene expression analysis even on partially degraded RNA may be desirable, e.g. in ancient, necrotic, clinical or biopsy samples. Yet, little is known about the possibility of obtaining reasonable qRT-PCR data from RNA samples with impaired RNA quality. Until today there are no statistically confirmed studies at which threshold RNA integrity is useless for quantitative downstream applications. With that in mind, and with the aim of anticipating future standards, we identified and analyzed the effect of various artificial and standardized degraded RNA samples on the two most abundant relative quantification models: the delta-delta Ct (Livak and Schnittgen 2001) and the efficiency corrected Ct model (Pfaffl 2001). The procedure of normalizations with an internal reference mRNA standard can reveal the importance of a relative expression approach to exclude compounded errors by variation in RNA quality and quantity. A further aim of the study was to investigate whether a correlation exists between PCR performance, PCR efficiency, length of amplified product and quality of RNA.

Materials and methods

Sample preparation

Two experimental set-ups were processed. In the first experiment total RNA was prepared from eight different bovine tissue types [lymph node, colon, corpus luteum, caecum, spleen, abomasums, reticulum and white blood cells (WBC)] and two primary cell cultures (granulosa and kidney cells). In the second experiment total RNA extracted from bovine spleen, corpus luteum, liver and WBC was investigated.

RNA purification in both experiments was performed by a slightly modified phenol-based extraction method, using peqGOLD TriFast (PeqLab, Erlangen, Germany). 500 µl peqGOLD TriFast and 50 mg tissue (stored at –80°C) were added to impact-resistant 2 ml tubes (MP Biomedicals, Solon, OH) pre-filled with 200 mg specialized lysing matrix particles (Qbiogene, Morgan Irvine, CA). The samples were homogenized two times by mechanical disruption using the FastPrep 120 instrument at speed 6.0 for 30 s. (Qbiogene). RNA extraction was carried out in RNAse-free environment and the purified total RNA was eluted in RNAse-free water (Eppendorf, Hamburg, Germany). Nucleic acid concentrations were measured in triplicate at 260 nm by using the BioPhotometer (Eppendorf). Purity of the total RNA extracted was determined as the A_{260}/A_{280} ratio with expected values between 1.8 and 2.

Artificial total RNA degradation

In order to get RNA samples with different and standardized degradation levels, but with the identical transcriptome and tissue typical mRNA distribution, intact cellular RNA was artificially degraded either enzymatically via ubiquitary skin RNAses for 10 s, or by irradiation with ultraviolet-C radiation (UVC) at 200–280 nm (Kendro UV-C 30, Langenselbold, Germany). In the first experiment reticulum and lymph node$_{(e)}$ were treated enzymatically ($n = 2$). Lymph node$_{(p)}$, colon, corpus luteum, caecum, spleen, abomasums, WBC, kidney cells and granulose cells were treated physically ($n = 9$) by UV-C radiation (Kundu et al. 2004). In the second experiment only physical degradation via UVC was performed ($n = 4$). Depending on the type of tissue each sample was placed under a UVC lamp for a tissue specific period of time up to 120 min. For both experimentals, intact RNA samples from the identical tissue extraction were mixed in various ratios with degraded RNA samples to generate a RIN gradient. Each sample consists of 10–12 denaturation grades (in total 135 samples) and ranging from intact RNA (RIN >7.5) to highly degrade RNA (RIN <3).

RNA integrity number (RIN) algorithm analysis

For the rapid quantification of nucleic acids the Agilent 2100 Bioanalyzer (Agilent Technologies), a chip-based nucleic acid separation system was used. The Bioanalyzer utilizes a combination of micro-fluidics, capillary electrophoresis, and fluorimetry to determine RNA length, distribution and concentration. The RNA Nano 6000 LabChip kit (Agilent Technologies) was used together with a standardized RNA ladder (Ambion, Austin, TX, USA) for RNA analysis and quantification. Altogether 135 RNA samples with different total RNA degradation levels were investigated in triplicates ($n = 405$).

One-step qRT-PCR

In the first experiment the expression levels of four representative genes (18S, 28S and β-actin and IL-1β) were measured. High abundant ribosomal 18S and 28S rRNA subunits, medium abundant β-actin and low abundant IL-1β genes were used to cover all possible abundance levels of a normal distributed transcriptome. Quantification was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 50 ng total RNA of various RNA degradation levels were used as template in the Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia). For ribosomal subunits, 18S and 28S rRNA, 1:10.000 dilutions were used in qRT-PCR. The real-time qRT-PCR master-mix was prepared as follows: 5 µl 2X

SYBR Green Reaction Mix, 0.5 µl forward primer (10 pmol), 0.5 µl reverse primer (10 pmol) and 0.2 µl SYBR Green One-Step Enzyme Mix (Invitrogen).About 6.2 µl of master mix was filled into a tube and a 3.8 µl volume of total RNA was added as PCR template. Bovine sequence-specific primers were synthesized commercially (MWG Biotech, Ebersberg, Germany) as shown in Table 1. A four-step experimental run protocol was used: (1) reverse transcription (10 min at 50°C); (2) denaturation program (5 min at 95°C); amplification and quantification program repeated 40 times (15 s at 95°C; 30 s at 60°C with a single fluorescence measurement; 20 s at 68°C); (3) melting curve program (60–99°C with a heating rate of 0.5°C/s and a continuous measurement); (4) cooling program down to 40°C.

Cycle threshold (Ct) and amplification efficiency

Ct values and qPCR efficiency were computed with the *''comparative quantitation''* method in the real-time qPCR Analysis Software, version 6.0 (Corbett Life Science). The Ct value is defined as the point at which the fluorescence rises above the background fluorescence (Pfaffl et al. 2002).

Relative quantification

The Ct of three target genes (18S, β-actin and IL-1β) was normalized to the chosen reference gene Ct of 28S rRNA. In the first relative quantification approach no efficiency correction was performed according to the delta-delta Ct model (Livak and Schnittgen 2001) shown in Eqs. 1 and 2.

$$R = 2^{-[\Delta Ct\,\text{sample} - \Delta Ct\,\text{control}]}, \quad (1)$$

$$R = 2^{-\Delta\Delta Ct}. \quad (2)$$

In the second approach the efficiency correction was implemented additionally (Eqs. 3, 4) and the advantages of an efficiency corrected quantification model were applied (Pfaffl 2001). The derived ratio values describe the relative expression change of the target gene relative to the 28S reference gene expression:

Table 1 Description of used primers

Gene	Primers	Length [bp]	Annealing temperature [°C]
Study 1			
18S rRNA	for: GAG AAA CGG CtA CCA CAT CCA rev: GAC ACt CAG CtA AGA GCA TCG A	338	60
28S rRNA	for: TAA CAA GCC GGT AGC CCA CG rev: GCA AGG GCt CtT GAT GGC AGA	238	60
β-actin	for: AAC TCC ATC ATG AAG TGT GAC G rev: GAT CCA CAT CtG CtG GAA GG	202	60
IL-1β	for: TTC TCt CCA GCC AAC CtT CAT T rev: ATC TGC AGC TGG ATG TTT CCA T	198	60
Study 2			
β-actin 50	for: ATC CtG CGT CtG GAC CtG rev: ACG CtC CGT GAG GAT CtT C	66	60
β-actin 100	for: GAG CGA GGC TAC AGC TTC A rev: CAT CtC CtG CtC GAA GTC CA	99	60
β-actin 200	for: GGC ATC GTG ATG GAC TCC rev: GAG CtT CtC CtT GAT GTC ACG	201	60
β-actin 400	for: TCt ACA ACG AGC TCC GTG TG rev: GAG CtT CtC CtT GAT GTC ACG	380	60
β-actin 600	for: GGC ATC GTG ATG GAC TCC rev: TCt GCt GGA AGG TGG ACA G	616	60
β-actin 800	for: TCt ACA ACG AGC TCC GTG TG rev: TCt GCt GGA AGG TGG ACA G	795	60
β-actin 950	for: GTC TTC CCG TCC ATC GTG rev: TCt GCt GGA AGG TGG ACA G	976	60

$$R = \frac{(E_{\text{target}})^{\Delta \text{Ct target (control}-\text{sample)}}}{(E_{\text{ref}})^{\Delta \text{Ct ref (control}-\text{sample)}}}, \quad (3)$$

$$R = \frac{(E_{\text{ref}})^{\text{Ct sample}}}{(E_{\text{target}})^{\text{Ct sample}}} \div \frac{(E_{\text{ref}})^{\text{Ct control}}}{(E_{\text{target}})^{\text{Ct control}}}. \quad (4)$$

PCR product length

In the second experiment sample RNA was diluted to a final concentration of 20 ng/µl. Seven PCR primer sets were designed using HUSAR software and synthesized by MWG Biotech (Ebersberg) to amplify different sequence fragments of β-actin (Table 1). Primer characteristics like primer dimer, self binding or false binding ability were minimized. GC content and PCR annealing temperature of all primer sets were adjusted and optimized to constant values. The one-step qRT-PCR was performed and expression levels of β-actin were measured with the standardized protocol as described above. Some minor changes were introduced in the time of denaturation depending on product length. For the product length of 800 and 950 bp attuned to 20 s. Elongation time at 68°C was slightly changed and adapted to different product lengths: 10 s for 50 bp, 20 s for 100 and 200 bp, 30 s for 400 bp, 60 s for 800 and 950 bp. In order to prevent inter-assay variation, samples with the same primer set were always amplified within one run. To ensure that PCR products from qRT-PCR had the expected size, an agarose gel electrophoresis was performed (gel not shown).

Statistical interference

All statistical analyses were performed by using Sigma Stat 3.0 (SPSS Inc., Chicago, IL). The determined *P*-values of the statistical significance were analyzed using linear regression and coefficient of determination (R^2). Significance of linear regression was tested by Student's *t*-test, by testing the slope to be different from zero. Coefficients were recorded when significant at $P < 0.05$. Higher significance levels were considered when available. All data were plotted in Sigma-Plot 8.0 (SPSS) and Microsoft Excel (Microsoft, Redmond, WA).

Results

RNA quality assessment and RNA degradation levels

Isolated total RNA quality was verified by an average A_{260}/A_{280} ratio of 1.88 (range 1.75–2.01). An A_{260}/A_{280} ratio greater than 1.8 is usually considered an acceptable indicator of good RNA (Sambrook et al. 1989). No phenolic contamination or background absorption was reported via the A_{260}/A_{230} ratio. All 135 artificial total RNA degradation gradient samples, were measured in triplicates in the Bioanalyzer 2100, and ranged from integer to degraded quality levels: RIN 7.3–9.5 for integer down to RIN 1.1–3.0 for degraded RNA (total measurements $n = 405$). Furthermore, the dependency of the RNA quality on tissue type, WBC and cell-lines was determined. The average RIN for solid tissues ranged between 5.4 and 9.6, whereas tissues or organs with high content of connecting tissue, for example in the gastrointestinal tract, showed higher variations in RIN values. In cell culture and WBC the RIN ranged between 8.4 and 9.6 with low experimental variance (Fleige and Pfaffl 2006).

Confirmation of primer specificity

The expression levels of four genes (18S, 28S, β-actin and IL-1β) were measured in all RNA quality aliquots. For each analyzed gene a melting curve analysis was performed. All investigated qRT-PCR products showed only single peaks and no primer-dimer peaks or artifacts. The specificity of qRT-PCR products was documented with high resolution gel electrophoresis and resulted in a single product with the desired length (not shown).

Gene expression profiling versus RNA integrity

To determine how qRT-PCR is affected by the integrity of the starting RNA, we compared Ct

Table 2 Correlation between RNA integrity and gene expression

	18S rRNA		28S rRNA		β-actin		IL-1β	
	R^2	P	R^2	P	R^2	P	R^2	P
UV degradation								
Lymph node$_{(p)}$	0.911	< 0.001	0.946	< 0.001	0.812	< 0.001	0.903	< 0.001
	$y = -0.633x + 17.38$		$y = -0.492x + 15.38$		$y = -0.794x + 15.06$		$y = -0.674x + 24.68$	
Colon	0.541	< 0.001	0.911	< 0.001	0.72	< 0.001	0.725	< 0.001
	$y = -0.873x + 26.16$		$y = -0.337x + 14.21$		$y = -0.604x + 15.57$		$y = -0.847x + 24.65$	
Corpus luteum	0.832	< 0.001	0.948	< 0.001	0.912	< 0.001	0.885	< 0.001
	$y = -1.568x + 26.83$		$y = -0.489x + 16.45$		$y = -0.801x + 15.47$		$y = -0.907x + 27.95$	
Caecum	0.743	< 0.001	0.842	< 0.001	0.965	< 0.001	0.947	< 0.001
	$y = -0.614x + 18.91$		$y = -0.589x + 16.45$		$y = -0.901x + 16.99$		$y = -0.608x + 23.07$	
Spleen	0.606	< 0.001	0.686	< 0.001	0.673	< 0.001	0.894	< 0.001
	$y = -1.544x + 25.39$		$y = -0.413x + 14.74$		$y = -0.704x + 15.79$		$y = -0.591x + 21.48$	
Abomasum	0.752	< 0.001	0.876	< 0.001	0.776	< 0.001	0.868	< 0.001
	$y = -1.251x + 22.18$		$y = -0.745x + 16.35$		$y = -1.199x + 20.69$		$y = -0.740x + 29.78$	
WBC	n.a.	n.a.	0.534	< 0.05	0.746	< 0.001	0.799	< 0.001
	n.a.		$y = -0.243x + 27.49$		$y = -0.923x + 21.61$		$y = -0.751x + 27.25$	
Kidney cells	0.907	< 0.001	0.581	0.002	0.833	< 0.001	0.901	< 0.001
	$y = -0.346x + 26.88$		$y = -0.191x + 13.624$		$y = -1.617x + 22.04$		$y = -0.959x + 29.42$	
Granulosa cells	0.182	< 0.001	0.248	< 0.001	0.776	< 0.001	0.868	< 0.001
	$y = -0.114x + 13.22$		$y = -0.104x + 25.67$		$y = -1.199x + 20.69$		$y = -0.740x + 29.78$	
Enzymatic degradation								
Reticulum	0.28	< 0.001	0.352	< 0.001	0.803	< 0.001	0.717	< 0.001
	$y = -0.518x + 22.53$		$y = -0.284x + 13.44$		$y = -0.582x + 13.26$		$y = -0.133x + 21.97$	
Lymph node$_{(e)}$	0.579	< 0.001	0.265	< 0.01	0.594	< 0.001	0.842	< 0.001
	$y = -0.231x + 14.37$		$y = -0.038x + 12.05$		$y = -0.092x + 8.96$		$y = -0.088x + 19.59$	

Impact of RNA integrity (shown as RIN $= x$) on *cycle threshold* (Ct shown as Ct $= y$). Data are representing as linear regression, coefficient of determination (R^2) and the P-values of regression. Four different genes (18S, 28S, β-actin and IL–1β), were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

levels or single-run PCR efficiency with the RNA integrity (Tables 2 and 3). Therefore, RIN numbers were linearly regressed with Ct or efficiency values. Each amplified gene was tissue specifically influenced by the RNA integrity, reflected by the level of significance (P-value) and the Pearson correlation coefficient (R^2) in the applied linear regressions. This demonstrates an incomprehensible tissue-matrix-effect between RNA integrity and type of tissue and the analyzed transcript (cited the lymph-node as an example in Fig. 1). The expression data demonstrates that a high-quality, intact RNA will result in a high expression level (low Ct) and a less-quality RNA results in low expression level (high Ct). The mean coefficient of determination in all regressed genes and tissues ($R^2 > 0.812$) shows that there is a causally determined high correlation between RIN and the Ct ($n = 53$). The determined P-values provide a statistical significance, in almost all datasets of $P < 0.001$.

28S/18S rRNA ratio

In a further sub-study all 28S/18S ratios were compared with the RIN. No clear trend of RNA quality compared to 28S/18S ratios could be found, therefore the 28S/18S ratio data are not presented.

Real-time PCR amplification efficiencies

Our research into RNA integrity effect on the single-run qRT-PCR efficiency has been carried out similarly to the above mentioned tissues and artificial RNA qualities. The efficiency variations for the lymph node were diagrammed in Fig. 2. Each analyzed tissue turned out to have significant tissue specific qPCR amplification efficiencies.

Table 3 Correlation between RNA integrity and real-time PCR efficiency

Tissue	18S rRNA		28S rRNA		β-actin		IL-1β	
	R^2	P	R^2	P	R^2	P	R^2	P
UV degradation								
Lymph node$_{(p)}$	0.009	0.58	0.074	0.11	0.004	0.74	0.348	<0.001
	$y = -0.002x + 1.73$		$y = 0.001x + 1.75$		$y = -0.0009x + 1.77$		$y = 0.017x + 1.68$	
Colon	0.036	0.33	0.069	0.13	0.0002	0.94	0.292	<0.001
	$y = -0.005x + 1.53$		$y = 0.004x + 1.74$		$y = -0.0003x + 1.79$		$y = 0.013x + 1.69$	
Corpus luteum	0.212	<0.01	0.024	0.37	0.004	0.06	0.008	0.59
	$y = -0.008x + 1.72$		$y = 0.001 + 1.75$		$y = 0.004x + 1.75$		$y = 0.002x + 1.78$	
Caecum	0.174	<0.05	0.003	0.76	0.231	<0.01	0.074	0.11
	$y = -0.012x + 1.67$		$y = -0.001 + 1.76$		$y = -0.011x + 1.83$		$y = -0.005x + 1.78$	
Spleen	0.048	0.21	0.049	0.19	0.536	<0.001	0.137	<0.05
	$y = 0.011x + 1.52$		$y = 0.004 + 1.74$		$y = 0.017x + 1.72$		$y = -0.006x + 1.81$	
Abomasum	0.020	0.41	0.009	0.57	0.477	<0.001	0.094	0.07
	$y = -0.001x + 1.63$		$y = -0.001 + 1.75$		$y = -0.012x + 1.81$		$y = -0.006x + 1.79$	
WBC	n.a.	n.a.	0.744	<0.001	0.011	0.46	0.088	<0.01
	n.a.		$y = -0.065x + 1.99$		$y = -0.002x + 1.81$		$y = -0.004x + 1.82$	
Kidney cells	0.625	<0.05	0.315	0.051	0.218	0.108	0.161	0.174
	$y = -0.016x + 1.85$		$y = 0.006x + 1.69$		$y = 0.002x + 1.76$		$= -0.006x + 1.86$	
Granulosa cells	0.447	<0.01	0.228	<0.01	0.523	<0.001	0.012	0.66
	$y = 0.002x + 1.69$		$y = 0.020x + 1.39$		$y = 0.015x + 1.63$		$y = 0.003x + 1.76$	
Enzymatic degradationr								
Reticulum	0.424	<0.001	0.039	0.28	0.024	0.39	0.0003	0.93
	$y = -0.024x + 1.62$		$y = -0.002x + 1.76$		$y = -0.002x + 1.81$		$y = 0.0003x + 1.85$	
Lymph node$_{(e)}$	0.41	0.02	0.182	<0.01	0.113	<0.05	0.05	0.19
	$y = 0.001x + 1.70$		$y = 0.005x + 1.75$		$y = 0.005x + 1.72$		$y = 0.004x + 1.77$	

Impact of RNA integrity (shown as RIN $= x$) on *qPCR efficiency* (shown as $E = y$). Data are shown as linear regression, coefficient of determination (R^2) and the P-values of regression. Four different genes (18S, 28S, β-actin and IL–1β), were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

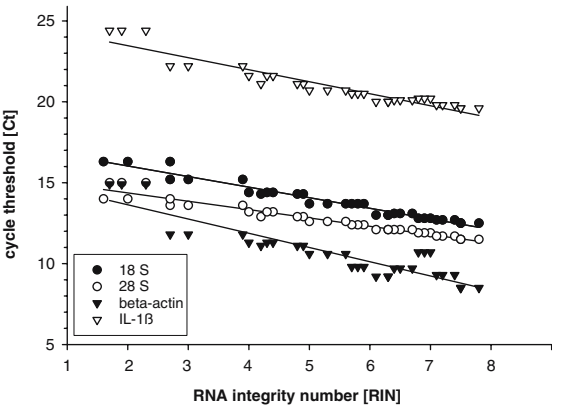

Fig. 1 Correlation between crossing point and RIN Distribution of the RT-PCR absolute expression measured on 12 RNA aliquots in triplicates ($n = 36$) from lymph node (cited as an example for all tested tissues and cell cultures) using 18S, 28S, β-actin and IL-1β. The linear regression lines are indicated and shown in detail in Table 2

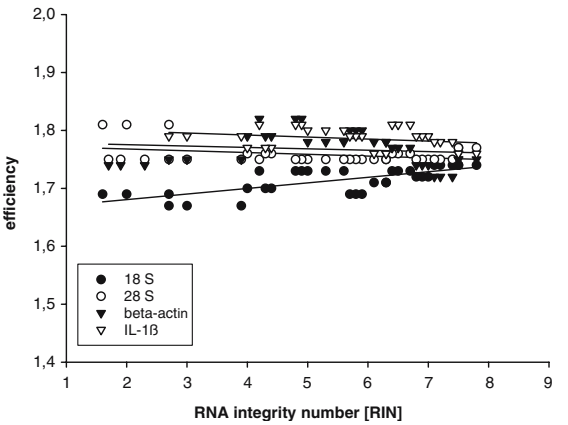

Fig. 2 Single-run PCR efficiency versus RIN Distribution of the RT-PCR efficiency measured on 12 RNA aliquots in triplicates ($n = 36$) from lymph node (cited as an example for all tested tissues and cell cultures) using 18S, 28S, β-actin and IL-1β. The linear regression lines are indicated and shown in detail in Table 3

Some tissues had higher amplification efficiencies with increasing RNA integrity, shown as positive coefficient of regression. Looking over all analyzed genes and tissues (Table 3) the RIN effect on qPCR efficiency was minor, compared to the influence on Ct (Table 2). The data demonstrates that amplification efficiency is highly dependent on the incomprehensible tissue-matrix-effect (represented by the intercept of the regression equation) and only minor affected by the RNA integrity itself (represented by the slope of linear regression). However, the coefficient of regression of the amplification efficiency data showed significance for some tissues and genes.

Effect of normalization

To test the effect of normalization by an internal reference gene, expression results of 18S, β-actin and IL-1β were normalized to the reference gene 28S rRNA. 28S rRNA was chosen as an optimal reference gene, because it showed the lowest variations during the performed RNA degradation study. 28S rRNA expression too, showed the lowest slope in the Ct and efficiency analysis (Tables 2 and 3). The relative quantification alteration in expression of the target gene relative to the reference gene was performed in two ways: first according to the delta-delta Ct method (Livak and Schmittgen 2001), and second by the single-run-specific efficiency-corrected relative expression model (Pfaffl 2001, LightCycler Relative Quantification Software, Version 1.0). For both models the RIN values were regressed versus the relative expression ratios. In the first model an optimal amplification efficiency of two ($E = 2$) was assumed. For nearly all genes and tissues we could show a significant effect on relative expression level depending on the RNA quality (Table 4). In the advanced calculation

Table 4 Correlation between RNA integrity and delta-delta Ct model

Tissue	18S rRNA		β-actin		IL–1ß	
	R^2	P	R^2	P	R^2	P
UV degradation						
Lymph node$_{(p)}$	0.764 $y = 0.071x + 0.03$	< 0.001	0.465 $y = 0.803x + 0.20$	< 0.001	0.666 $y = 0.001x + 0.01$	< 0.001
Colon	0.226 $y = 0.002x - 0.01$	< 0.001	0.412 $y = 0.161x + 0.28$	< 0.001	0.841 $y = 0.001x - 0.01$	< 0.001
Corpus luteum	0.766 $y = 0.064x - 0.16$	< 0.001	0.770 $y = 1.128x + 2.38$	< 0.001	0.768 $y = 0.001x + 0.01$	< 0.001
Caecum	0.263 $y = 0.024x + 0.05$	< 0.001	0.698 $y = 0.541x - 1.130$	< 0.001	0.214 $y = 0.003x + 0.01$	< 0.001
Spleen	0.307 $y = 0.068x - 0.20$	< 0.001	0.564 $y = 0.241x + 0.34$	< 0.001	0.706 $y = 0.003x + 0.01$	< 0.001
Abomasum	0.388 $y = 0.029x - 0.01$	< 0.001	0.624 $y = 0.048x + 0.057$	< 0.001	0.0005 $y = -0.0002x + 0.000$	< 0.001
WBC	n.a. n.a.	n.a.	0.759 $y = 560.16x - 1580$	< 0.001	0.777 $y = 3.795x - 9.47$	< 0.001
Kidney cells	0.741 $y = 0.0002x + 0.000$	< 0.001	0.640 $y = 1.205x - 0.84$	< 0.001	0.718 $y = 0.0002x - 0.000$	< 0.001
Granulosa cells	0.707 $y = 0.035x - 0.13$	< 0.001	0.383 $y = 1.212x - 0.09$	< 0.001	0.399 $y = 0.001x - 0.000$	< 0.001
Enzymatic degradation						
Reticulum	0.026 $y = -0.005x + 0.09$	0.25	0.703 $y = 0.566x + 0.71$	< 0.001	0.198 $y = -0.001x + 0.01$	< 0.001
Lymph node$_{(e)}$	0.398 $y = 0.056x + 0.13$	< 0.001	0.327 $y = 0.394x + 0.35$	< 0.001	0.399 $y = 0.001x + 0.01$	< 0.001

Impact of RNA integrity (shown as RIN $= x$) on relative expression ratio, calculated according the equation delta-delta Ct model 2^Ct(RG)-Ct(TG) (shown as y value). Data are shown as linear regression ($n = 36$), coefficient of determination (R^2) and the P-values of regression. Three efficiency-corrected relative expression ratios are shown (18S, β-actin and IL-1β) and 28S rRNA was used as reference gene. Expression ratios were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

model, according to the efficiency corrected model regressed data are shown in Table 5. Significant, positive correlations (mostly $P < 0.001$) and regression coefficients between the RNA integrity and the relative expression of the quantified target genes were determined in the sample-specific efficiency-corrected relative quantification (shown as $E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ values). To proof the feasibility of this model a intact RNA at RIN value 8 ($E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ = 4.65, defined as sample) and a degraded RNA at RIN value 2 ($E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ = 1.16, defined as control) were compared for the β-actin mRNA expression (Fig. 3). The defined sample contained fourfold more β-actin molecules than the control, meaning around 75% β-actin mRNA was degraded.

Effect of length of the amplified product

To test the influence of PCR product length, a gradient with several steps of intact RNA down to degraded RNA was examined with seven primer sets, amplifying qRT-PCR products of various lengths up to 976 bp. To amplify the sequence of β-actin in different tissues and varying RNA integrity levels, seven primer sets were used. In Fig. 4 it is clearly visible for all product length that the crossing point is shifted towards lower cycle numbers using intact total RNA. The best repeatability could be attained in quadrant IV with a RIN value higher than five (even better over eight), and a qRT-PCR product length lower than 200 bp. These results could be confirmed for all analyzed tissues and genes. Quadrant I and III showed low RNA quality having RIN lower than

Table 5 Correlation between RNA integrity and efficiency-corrected relative expression ratio

Tissue	18S rRNA		β-actin		IL-1β	
	R^2	P	R^2	P	R^2	P
UV degradation						
Lymph node$_{(p)}$	0.129 $y = 0.042x + 0.45$	< 0.05	0.543 $y = 0.581x + 0.001$	< 0.001	0.311 $y = 0.0008x + 0.01$	< 0.001
Colon	0.209 $y = 0.053x + 0.000$	< 0.01	0.193 $y = 0.153x + 0.18$	< 0.01	0.157 $y = 0.002x + 0.004$	< 0.05
Corpus luteum	0.659 $y = 0.105x + 0.04$	< 0.001	0.476 $y = 0.397x + 2.30$	< 0.001	0.027 $y = 0.0003x + 0.004$	0.34
Caecum	0.289 $y = 0.164x + 0.17$	< 0.001	0.606 $y = 0.345x + 0.000$	< 0.001	0.215 $y = 0.003x + 0.008$	< 0.05
Spleen	0.603 $y = 0.155x + 0.000$	< 0.001	0.110 $y = 0.104x + 0.59$	< 0.05	0.538 $y = 0.009x + 0.01$	< 0.001
Abomasum	0.333 $y = 0.082x + 0.19$	< 0.001	0.677 $y = 0.073x + 0.02$	< 0.001	0.137 $y = 0.0001x + 0.00$	< 0.05
WBC	n.a. n.a.	n.a.	0.006 $y = 0.946x + 45.71$	0.76	0.031 $y = 0.445x + 1.06$	0.47
Kidney cells	0.642 $y = 0.0003x + 0.01$	< 0.001	0.565 $y = 0.664x + 0.001$	< 0.001	0.567 $y = 0.0001x + 0.001$	< 0.001
Granulosa cells	0.078 $y = 0.082x + 0.38$	0.22	0.432 $y = 0.566x + 0.35$	= 0.001	0.323 $y = 0.002x + 0.003$	< 0.01
Enzymatic degradation						
Reticulum	0.208 $y = 0.047x + 0.15$	< 0.01	0.613 $y = 0.288x + 0.62$	< 0.001	0.153 $y = -0.0004x + 0.01$	< 0.05
Lymph node$_{(e)}$	0.299 $y = 0.089x + 0.33$	< 0.001	0.195 $y = 0.324x + 6.25$	< 0.01	0.032 $y = 0.0004x + 0.01$	0.29

Impact of RNA integrity (shown as RIN = x) on efficiency-corrected relative expression ratio, calculated according the equation $E_{(RG)}$^$Ct_{(RG)}$/$E_{(TG)}$^$Ct_{(TG)}$ (shown as y value). Data are shown as linear regression ($n = 36$), coefficient of determination (R^2) and the P-values of regression. Three efficiency-corrected relative expression ratios are shown (18S, β-actin and IL-1β) and 28S rRNA was used as reference gene. Expression ratios were analyzed in 11 tissues, sorted by various type of degradation, either UV degradation ($n = 9$) or enzymatic degradation ($n = 2$)

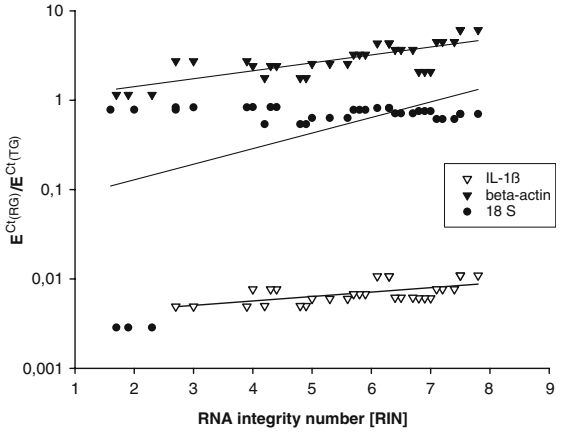

Fig. 3 Single-run efficiency-corrected relative expression ratio $E_{(RG)}{}^{\wedge Ct(RG)}/E_{(TG)}{}^{\wedge Ct(TG)}$ ratio versus RIN Distribution measured on 12 RNA aliquots in triplicates ($n = 36$) from lymph node (cited as an example for all tested tissues and cell cultures) using 28S as reference gene. The linear regression lines are indicated and shown in detail in Table 4

five and high variability in qRT-PCR results. In quadrant II high quality RNA was used, but high amplicon size resulted in late and highly variable Ct, and consequently in inefficient reaction with low PCR efficiency (no figure shown). High Ct values for 800 and 950 bp may result from inefficient amplification as from too long qRT-PCR products and the applied stringent cycle conditions.

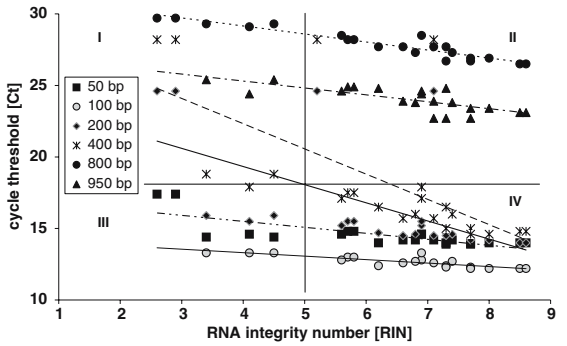

Fig. 4 Relationship between RNA integrity and length of the amplified product Integrity of 23 bovine corpus luteum RNA sample (cited as an example for all tested tissues) profiles was scored using the RIN software. Cycle threshold (Ct) values in dependence on amplicon length and RNA integrity (RIN). Comparative analysis was done using β-actin with different length of the amplified product. Graph is divided in four quadrants (I–IV)

Discussion

Intact RNA is essential for many molecular biotechnology techniques used in gene expression studies. It is universally accepted that RNA purity and integrity are of foremost importance to ensure reliability and reproducibility of qRT-PCR. Despite this valid assumption, there has been minor experimental and statistical proven data to verify this assertion. Spectrophotometer analysis of RNA in particular has been widely accepted as an important quality assurance measures for RT-PCR and microarray experiments (Baelde et al. 2001). The spectrophotometer abnortancy measurement has long been used as a criterion for assessing contamination of RNA samples throughout the development of molecular biology. The A_{260}/A_{280} and A_{260}/A_{230} ratios reflect RNA purity but are not informative regarding the integrity of the RNA (Mueller et al. 2000). A_{260}/A_{280} ratios higher than 1.8 are indicative of limited protein contaminations, whereas low A_{260}/A_{230} ratios are indicative of residual contamination by organic compounds such as phenol, sugars or alcohol, which could be highly detrimental to downstream applications (Sambrook et al. 1989). Today micro-fluidic capillary electrophoresis are more and more used for RNA quality and quantity assessments, particularly in the gene expression profiling platforms (Mueller et al. 2000; Lightfood 2002). From the shape of the electropherogram and the dominant ribosomal RNA subunits peaks, the software automatically generates the 28S/18S rRNA ratio and in newer software versions as well the RIN value (Mueller 2004; Schroeder et al. 2006). The RNA degradation is a gradual process and this is shown in a decrease of the ribosomal 28S/18S rRNA ratio and an increasing base-line signal between the two ribosomal peaks and the 5S rRNA peak. The 28S/18S ratio is calculated automatically, but it may not be used as a gold standard for assessing RNA integrity because of its high variability (CV 19–24%) (Imbeaud et al. 2005; Fleige and Pfaffl 2006; Schroeder et al. 2006). Our dataset could confirm this finding in 11 analyzed tissues showing a high 28S/18S ratio variations (CV 32 ± 18%; $n = 405$). No significant correlation

between the 28S/18S rRNA ratio and real-time RT-PCR performance could be shown. Therefore the 28S/18S rRNA ratio could not be recommended as useful indicator of RNA integrity.

Effect of RNA integrity on PCR performance

Bustin and Nolan (2004) proposed performing a RNA quality control prior to downstream quantification assays, especially if one aims to accurately quantify small expression differences (Perez-Novo et al. 2005). The examination of the RNA integrity before use in different applications enabled to compare experiments and classify the significance of final gene expression results (Imbeaud et al. 2005). Therefore, we focused the influence of degraded RNA on the performance of qRT-PCR. A biologically *''normal and integer''* transcriptome of distinct bovine tissues or cell-lines were isolated and artificially degraded factiously by enzymatic digest or with ultraviolet light UVC (Kundu et al. 2004). A gradient with several steps of intact down to degraded RNA was researched by real-time qRT-PCR expression analysis. A significant negative relationship between the RIN and Ct for all tested samples is proven ($P < 0.001$). A conclusion from Auer et al. (2003) aforesaid, that degradation does not prelude micro array analysis if comparison is done using samples of comparable RNA integrity. Imbeaud et al. (2005) and Schroeder et al. (2006) showed the direct influence of RNA integrity on the absolute gene expression results. We could confirm mentioned studies using an RNA-integrity gradient in two prominent relative quantification models.

Impact of RIN on relative quantification

Normalization by an internal reference gene reduces or even diminishes tissue derived effects on qRT-PCR (Wittwer et al. 1997). Specific errors in the mRNA quantification procedure are easily compounded by any variation in the amount of starting material between samples (Gottwald et al. 2001) and on variation in the RNA integrity (own statement). A normalization of target genes with an endogenous expressed reference standard is strictly recommended. The applied standard should not be regulated or at least be minor regulated, like in the applied study the 28S rRNA expression. Furthermore the sensitivity to RNA degradation must be pointed out, because reference genes varying in theirs sensitivity (Perez-Novo et al. 2005). An accurate and relevant normalization to some internal standard is obligatory for biologically meaningful mRNA quantification (Bustin et al. 2005). With that prospect in mind, we correlate the RIN to normalized expression level values, normalized by an internally expressed reference gene, according to the two most abundant models: the delta-delta Ct method (Livak and Schmittgen 2001) and the efficiency-corrected model (Pfaffl 2001). It is well established that small efficiency differences between target and reference gene generate false expression ratios, resulting in over- or under-estimation of the real initial mRNA amount (Pfaffl 2001).

Ct and qPCR amplification efficiencies were determined sample-specific automatically in each single qRT-PCR reaction. Therefore, a direct RNA integrity influence on qPCR efficiency could be measured directly in each qRT-PCR sample. This method of efficiency determination is very comparable to earlier described methods and based on the single sample analysis, using multiple algorithms in the *''real''* exponential phase of PCR (Tichopad et al. 2003). Minor efficiency differences were found within one analyzed tissues (represented by the slopes of the linear regression), and most stable for 28S rRNA. The analyzed tissue itself is mainly influencing the PCR amplification efficiency (represented by the intercept of regression equation). It is well known from previous publications that many unknown factors in sample and exogenous contaminants inhibit PCR (Wilson 1997). Those tissue-matrix-effects relevant in qRT-PCR can be compounds like hemoglobin, fat, glycogen, cell constituents, or DNA binding proteins. Additionally, exogenous contaminants such as glow powder, phenolic compounds from extraction or plastic ware can have an inhibiting effect on reverse transcription and/or on PCR performance. The existence of an incomprehensible tissue-matrix-effect makes is important

to determine the qPCR efficiency tissue-by-tissue and run-by-run, and correct for it according to established models (Pfaffl et al. 2002, LightCycler Relative Quantification Software, Version 1.0).

Normalization of expression data by an internal reference gene on the basis of varying RNA integrities, showed to be strong RIN dependent. Herein the single-run specific efficiency was added to the model, as shown in Eqs. 3 and 4, to result in an efficiency-corrected relative quantification model. To visualize the results, as shown after normalization, fourfold more β-actin mRNA could be found comparing highly intact to degraded RNA. Statements about importance of normalization and efficiency correction as specified above could certify herewith. Results demonstrate that innovative new quantification methods and normalization models can improve mRNA quantification.

Interrelation between RIN and length of amplified product

Furthermore, the length of the amplified product influences PCR efficiency such as primer length, annealing temperature, and secondary structure (Bustin and Nolan 2004; Perez-Novo et al. 2005). Quantitative RT-PCR involves analysis of smaller mRNA regions and is therefore more tolerant of partially degraded RNA. However, RNA integrity control is often not systematically performed prior to qRT-PCR analyses (Perez-Novo et al. 2005). An interesting question is, if there is an increasing influence on the PCR performance with both variables (RNA quality and length of amplified product). Fragmentation of long mRNA will result in a loss of the molecule for qPCR detection only if the RNA break occurs within the product sequence. This might be a rare event in only moderately degraded RNA (Schoor et al. 2003). Therefore, the sequence of β-actin was assessed in different tissues and by varying RNA integrity. The results of correlation between RIN and Ct fulfilled the expectation for all tested tissues, where Ct value is shifted to lower cycle number with increasing RIN for all product lengths. Similarly low quality RNA pointed a high variability in qRT-PCR expression results. We subdivided the graph in four quadrants, which show the best repeatability in quadrant IV with high RNA integrity (RIN > 5) and product length up to 200 bp. The length of the amplified product is a very important part for primer design. Late and highly variable Ct (quadrant II) is also be due to amplified product length over 400 bp and good RNA quality. Maximum amplicon size should not exceed 400 bp (ideally 80–150 bases). Smaller amplicon give more consistent results because PCR is more efficient and more tolerant of reaction conditions. The research into the relationship between RNA integrity and length of the amplified product onto PCR efficiency show no correlation. With regard to the efficiency of the PCR, those can affect by a number of variables like length of the amplicon, RNA secondary structure and primer quality (Bustin and Nolan 2004; Wong and Medrano 2005).

Conclusion

Our data suggest that RNA quality control prior to qRT-PCR assays is indispensable. Tissue sampling, RNA extraction and storage are very sensitive to RNA integrity and should be designed to keep RNA pure and intact. Total RNA samples of high quality (RIN > 8) can serve as an optimal template whereas for partly degraded RNA ($8 >$ RIN > 5) result in suboptimal qRT-PCR expression results. Degraded RNA interferes with PCR performance as such, expressed as Ct value, whereas PCR efficiency is minor effected by RNA integrity. PCR efficiency seems to be major affected by the tissue type and extraction procedure.

The delta-delta Ct and the efficiency corrected model are both sensitive to RNA integrity. Statements about importance of normalization could be confirmed by our investigations, consequently we commended an efficiency-corrected relative quantification strategy and normalization with an internally reference gene for every quantitative mRNA expression analyses. In view of the observed difference in gene expression

stability between intact and degraded RNA sample, we and other authors (Bustin and Nolan 2004; Auer et al. 2003) propose performing RNA quality control prior to downstream quantification assays. We can recommend a RIN value higher than five and a PCR product length up to 200 bp as a minimal requirement for a successful and reliable real-time RT-PCR quantification.

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Appendix III:

Fleige S, Preißinger W., Meyer HHD, Pfaffl MW (2007):

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

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

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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) contain 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 ollected 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

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 (s.e. 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 was exchanged against whey powder to guarantee a 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		

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) according to Mayer (1969) 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

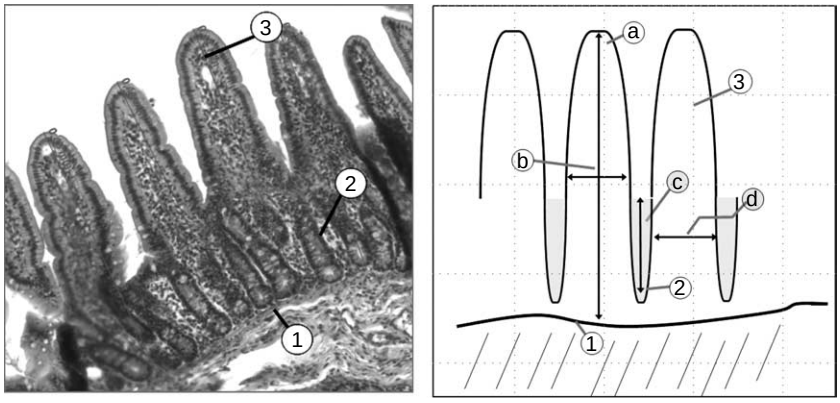

Figure 1 Morphological measurements in the intestine: (1) lamina muscularis mucosae; (2) crypt of Lieberkuhn; (3) villus. Measurements in the small and large intestine were combined pictured in the diagram. Small intestine: (a) villus height (from the tip of the villus to the lamina muscularis mucusae; (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).

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 ($P < 0.1$).

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 (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 n = 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

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*.

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Appendix IV:

Fleige S, Preißinger W., Meyer HHD, Pfaffl MW (2007):

Lactulose: Effect on apoptotic- and immunological markers in the gastrointestinal tract of pre-ruminant calves.

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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 crypth 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 Milkibeef 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 biqutin	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 ration (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 ± SEM. All statistical analysis 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-1β, 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

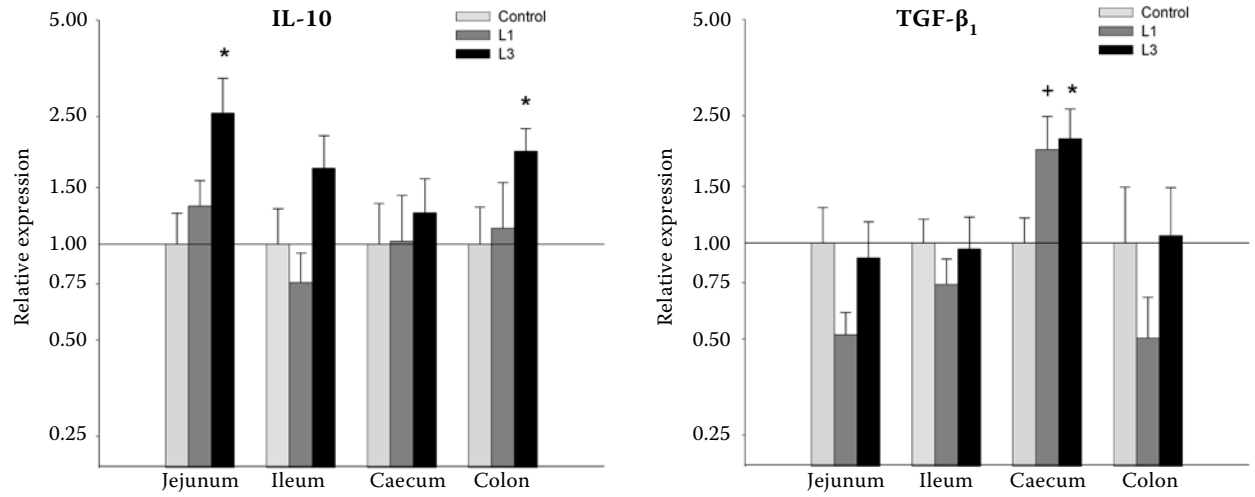

Figure 2. IL-10 and TGF-β_1 mRNA expression changes due to lactulose feeding. Data are presented as relative expression in means ± SEM (n = 14). Up-regulated genes are shown as values higher than 1 and down-regulated genes as values lower than 1. Significant effects of lactulose are marked with an asterisk (*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.

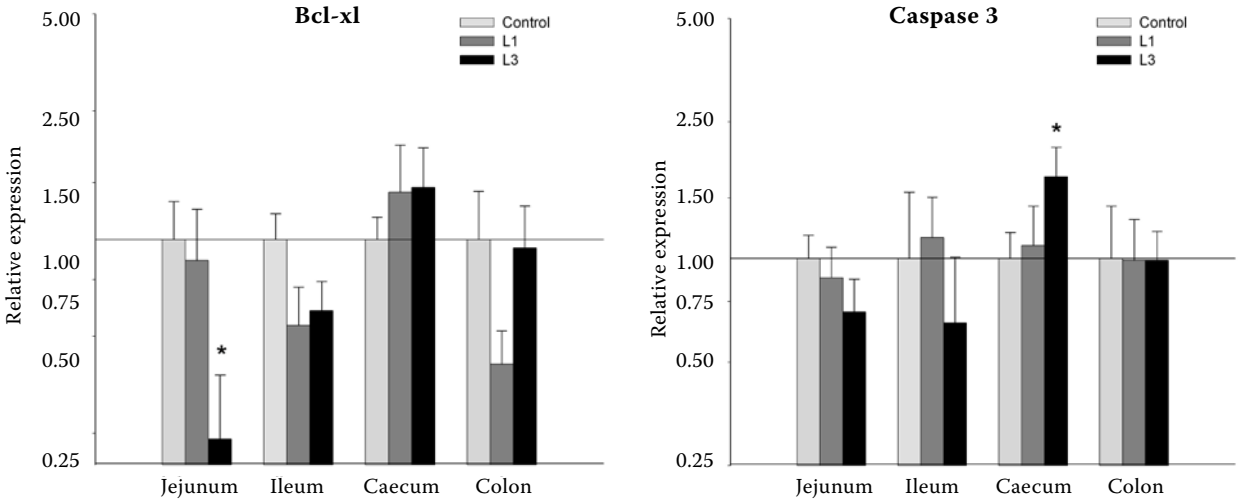

Figure 3. Effect of lactulose on the anti-apoptotic factor Bcl-xl and apoptotic factor caspase 3 in various intestinal calf tissues, compared to the untreated control group. Data are presented as relative expression in means ± SEM (n = 14). Up-regulated genes are shown as values higher than 1 and down-regulated 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-regualtory 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 upregulated 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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Appendix V:

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The immune – modulating effect of lactulose in combination with *Enterococcus faecium* in pre-ruminant calves.

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SUBMITTED

The immune-modulating effect of lactulose on *Enterococcus faecium* fed pre-ruminant calves.

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ABSTRACT Prebiotics and probiotics could represent an effective alternative to the use of synthetic antibiotics in nutrition. The mechanisms by which prebiotics affect the immune system are not investigated yet in detail. Many effects are attributed to the increase of the innate and the acquired immune response. The study was conducted to elucidate the long term effects of orally administered lactulose on the immune response of the intestinal tract of probiotic-fed calves. Pre-ruminant calves were randomized to three feeding groups: the control group (L0) was fed milk replacer containing *E. faecium*; a lactulose group (L1) containing additional 1% lactulose and a second lactulose group (L3) containing additional 3% lactulose. The mRNA expression of different cell activation markers, pro- and anti-inflammatory cytokines and immunoglobulin A Fc Receptor (IgA FcR), were investigated in ileum, mesenterial lymph node, spleen and white blood cells. A significant higher number of lymphocytes were detected in the L3 group. The expression results indicated that the transcription of IgA FcR in the ileal mucosa of the L3 treatment group increased significantly in male calves and also tended to increase in female calves. Furthermore a decrease of IL-10 and IFN-γ mRNA expression was observed in the ileum. CD4 presenting lymphocytes were decreased significantly in the ileum and mesenteric lymph node, whereas CD8 presenting lymphocytes were increased in blood. Other pro-inflammatory cytokines (IL-1β, IL-8 and TNF-α) and anti-inflammatory cytokines (TGF-β_1) did not show significant differences on mRNA level among treatment groups. The results indicate that additional lactulose feeding has an immune modulatory effect on the composition of T cell subsets in different immune compartments and minor effects on pro- and anti- inflammatory cytokine mRNA expression.

KEY WORDS lactulose, prebiotics, immunomodulation, T cell subsets, anti-inflammatory cytokines

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INTRODUCTION

Calf diseases – particularly diarrhoea and respiratory disease represent a serious economic impact on cattle up breeding companies (1). Prior to weaning, dairy calves fed with milk replacer (MR) are susceptible to many pathogens (1). Probiotics and prebiotics have been proposed as agents to manipulate the bacterial flora of the intestinal tract of animals, potentially reducing the incidence of disease. Today, a growing interest exists in the potential health-promoting benefits of prebiotics in farm animals. Multiple mechanisms of action for the beneficial effect prebiotics have been postulated, particularly by its enhancing effect on probiotic growth in the gut (2). The beneficial effects include the prevention of pathogenic bacteria growth, production of antimicrobial agents, stimulation of mucosal barrier function, and altering immunoregulation (3). The prebiotic lactulose is a synthetic disaccharide with several properties, including an anti-endotoxin effect and the potential to alter faecal floral patterns (4). Therefore it is predominantly used as an osmotic laxative (4). It is neither absorbed nor metabolized in the upper gastro intestinal tract (GIT), but it is degraded to organic acids by the bacterial flora of the proximal colon. Lactulose is a common used prebiotic in human health because of its stabilizing effect on the gut (5). At present, only few studies have investigated the direct effects of prebiotics on the immune system (6). Whether prebiotics modulate the immune response directly or indirectly, by affecting the composition of the intestinal flora (7), or by the fermentation to short chain fatty acids (SCFA), is still unknown. It was reported that the prebiotics inulin and oligofructose can modulate functions of the immune system, primarily the activation of immune cells in Peyer´s patches (PP) (8).

Another possibility to modulate the immune response is the combined use of prebiotics and probiotics (9) in order to improve survival of the probiotics by supplying a readily available and specific substrate for its fermentation (10). Especially probiotic effects are mediated through immune regulation, particularly through balance control of pro-inflammatory and anti-inflammatory cytokines. Probiotics mediate suppression of lymphocyte proliferation and cytokine production by T cells (11) and down-regulate the expression of pro-inflammatory cytokines as tumour necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1ß) and interferon gamma (INF-γ) (12). They interact with intestinal epithelia, and attenuate synthesis of inflammatory effector molecules elicited by diverse pro-inflammatory stimuli (13,14). Thus, probiotics and also prebiotics - that support the growth of probiotics - can be responsible for the unique tolerance of the gastrointestinal mucosa to pro-inflammatory stimuli. The used probiotic *E. faecium* belongs to the lactic acid bacteria and is a normal inhabitant of the gut flora of animals (15) with inhibitory effects against important enteropathogens (16). In several biological preparations *E. faecium* is used as feed additive and it was shown to stimulate the mucosal and the systemic immune system in dogs (17). Only a limited number of studies assessing the effect of lactulose on immune function have been published. The effect of a prebiotic administration or prebtiotic administration on probiotic-fed animals on the immune response was researched in previous studies with rats (18), mice (19), dogs (20) or pigs, neither with calves. Furthermore, results of several studies have been inconsistent and thus they were not directly comparable.

The purpose of the present study was to investigate the effect of a long term lactulose administration on the immune response in probiotic fed female and male calves. Therefore, we fed female and male calves with MR supplemented with *E. faecium* and different dose of lactulose. We assessed a broad spectrum of cytokines, markers of the T cell population and IgA FcR in different immune compartments.

MATERIALS AND METHODS

Experimental animals and treatment

42 Simmental calves from various farms were directly bought from the Simmental

breeding organisation (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 from 39 ± 2 days and gender was 50% male plus 50% female calves. During the feeding experiment, all calves were fed with MR from Milkibeef Top (Milkivit, Trouw Nutrition, Burgheim, Germany) containing 10^9 CFU (colony forming units) *E. faecium*. The control group (L0) was fed MR containing *E. faecium*; a lactulose group (L1) containing additional 1% lactulose and a second lactulose group (L3) containing additional 3% lactulose dry matter. Therefore, the MR for group L1 was mixed with 2.5% Lactusat and group L3 with 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/d 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 and blood samples

Tissue sampling: Samples of the middle part of ileum, mesenterial lymph node (mLN) and spleen were obtained at the time of slaughter. Immediately after collection the tissues were placed into individually labelled cryotubes and frozen in liquid nitrogen. Cryotubes were removed from liquid nitrogen in the laboratory and stored at -80°C until analysis. All animals had a healthy ileum, mLN and spleen with no visible pathological signs.

Blood sampling: 2x 15 ml blood samples were collected from the jugular vein using an EDTA and serum vacutainer tube (Greiner bio-one GmbH, Frickenhausen, Germany) following euthanasia. For the haematological analysis the blood samples were forwarded in EDTA vacutainer tubes to a veterinary laboratory (Vetmed Labor, Unterhaching, Germany). The haematocrit value and haemoglobin concentration, erythrocyte, thrombocyte and white blood cell (WBC) numbers were determined with the CELL-DYN 3700SL System (Abbott Diagnostika GmbH, Wiesbaden, Germany). Furthermore a blood smear was sending to obtain a differential white cell count. Blood smears were stained with May-Grünwald's eosin-methylene blue solution (Merck 1424) and Giemsa solution (Merck 9204) (22). The percentage of the different WBC subtypes, like granulocytes, lymphocytes, monocytes and atypical cells were determined. For WBC mRNA expression analysis EDTA blood was diluted 1/1 (v/v) with lysis buffer (830 mg NH_4CL, 3.7 mg Na-EDTA, 100 mg KCL in 100 ml H_2O pH 7.4) and centrifuged for 10 min at 220 g. The cell pellet was again suspended in lysis buffer and centrifugation was repeated. Supernatants of blood samples were discarded and leucocytes were transferred in 350 µl RNA extraction lysis buffer (Macherery-Nagel, Düren, Germany).

RNA extraction and RNA quality control

Total RNA from blood samples and each tissue sample (~50 mg) was extracted by using TriFast reagent (Peqlab, Erlangen, Germany) essential 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 (23). 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-PCRs 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: 5µl 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 in Tab. 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 sec at 95°C; annealing for 30 sec at 60°C; elongation for 20 sec at 68°C with a single fluorescence measurement); (iv) melting curve program (60-99°C with a heating rate of 0·5°C per sec and a continuous fluorescence measurement); (v) cooling program down to 40°C.

Data evaluation

Crossing Point (CP) and single run efficiency (E) values were achieved for each analyzed sample and gene using the Rotor-Gene 3000 software version 6.0 (Corbett Life Science). The relative expression changes were evaluated by using the single-run-specific efficiency-corrected relative expression model (24) (Fig.1). CP and E data for male and female calves (each n=7) were calculated separately. To assess the effect of the lactulose feeding on cytokine gene expression in the different tissues the relative expression ratio (R) was calculated, which is equal to a x-fold regulation. For normalization of each analyzed target gene (TG) the mean expression of the three reference genes (RG: ß-actin, GAPDH, Ubiquitin) was calculated. To factor the PCR efficiency into the analysis each analyzed sample was calculated apart with the sample specific efficiency. Cytokine mRNA expression is presented as relative expression whereas the appropriate mean control group (female or male control group) value was used as the calibrator. Values were expressed as means ± SEM. Analysis for the cytokine expression in all tissues studied was done by the 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. The statistical analysis was performed with Sigma Stat 3.0 (SPSS Inc. Chicago, IL, USA).

RESULTS

RNA quality

Isolated total RNA quality was verified by an average A260/A280 ratio of 1.88 (range 1.75–2.01). An A260/A280 ratio greater than 1.8 is usually considered an acceptable indicator of high quality RNA. No phenolic contamination or background absorption was reported via the A260/A280 ratio. The second quality control was done by the Bioanalyzer 2100. The total RNA quality of all samples studied ranged from RIN 7.2 to 9.3.

Haematology

The number of thrombocytes and lmyphocytes was affected by lactulose feeding. The number of thrombocytes decreased from 1203 ± 475 G/l in the control

group to 737 ± 318 G/l (P<0.05) in the L1 group. A significant higher number of lymphocytes from 56.4 ± 8.5% in the L3 group (P=0.02) versus 47.3 ± 10.5% in the control group was detected (Fig. 2). No differences in the haematocrit values, haemoglobin concentrations, and erythrocyte and leukocyte numbers were found among treatments.

Gene expression of pro- and anti-inflammatory cytokines

Lactulose affected a slight down-regulation of IL-10 in the ileum of the total L3 feeding group (P<0.05). The IL-10 relative expression (R) in the total L3 group was 0.29. A gender specific change of mRNA expression could determined for male calves of the group L3, which showed a down-regulation of 67% for IFN-γ and only a trend of down-regulation of 65% for IL-10 (0.05<P<0.10). No effect on the IFN-γ / IL-10 ratio was found. For further pro-inflammatory cytokines (IL-1β, IL-8, and TNF-α) and anti-inflammatory cytokines (TGF-β_1), no significant differences between mRNA expressions were found among treatment groups.

Alteration of T cell subgroups and T cell activation status

Lactulose induced mRNA expression changes for diverse CD markers (CD4, CD8, CD25 and CD69) are shown in table 2. The L1 treatment affects CD4 T-lymphocytes in the mLN and ileum of female calves. The CD4 expression in the mLN decreased about 51% (P<0.01) and about 60% in the ileum. In the same group a 2.1-fold increase of the CD8 T-lymphocyte expression could be observed in WBC. The CD25 expression in the mLN of male calves was reduced about 57% in L3 (P<0.05), while the CD69 expression significant decreased about 65% (P<0.05) in the spleen of male calves (L1).

IgA Fc Receptor mRNA expression

The treatments with lactulose show a significant effect on the mRNA expression of IgA FcR in the ileum. The male calves of L1 and L3 showed an up-regulation of IgA FcR, 2.6-fold (P<0·05) for L1 and 2-fold (0.05<P<0.10) for L3, respectively (Tab. 3).

DISCUSSION

The results demonstrate that the ileum and mLN are the tissues that are primarily affected by long term lactulose feeding. In addition, the supplementation of 1% lactulose has induced a significant immunomodulation in female calves, whereas the supplementation of 3% lactulose affects male calves in different immune compartments.

Moreover, the supplementation of 3% lactulose in male calves has reduced the production of IFN-γ. Considering that the humoral immunity is inhibited by IFN-γ we can assume that lactulose enhances the humoral immune response. The supplementation of lactulose in calves fed with *E. faecium* could have amplified the effect of the probiotic bacteria, which are able to enhance the humoral immune response and thereby promote the intestines immunologic barrier (11). In contrast to a number of animal studies (18,19) we could show a decrease of IL-10 mRNA expression in the ileum of the L3 group. Other studies with mice or rats demonstrated that consuming prebiotics like fructooligo-saccharide or inulin enhanced IFN-γ and IL-10 production by PP cells and that the combined use of pre- and probiotics abrogated the strong effect of prebiotics on PP cytokine production (19). A combined application of probiotics and prebiotics could have different effects from those of the individual supplements. In rats, a probiotic treatment modestly affected immune functions, whereas systemic immuno-modulatory effects were observed by a combined application of pre- and probiotic (18).

Immunologic responses to pathogens are mediated by the IgA FcR. The receptor is a transmembrane glycoprotein presented on the surface of myeloid lineage cells such as neutrophils, monocytes, macrophages and eosinophils, where it mediates immunologic responses to pathogens (25). Ligation of FcRs by Ig-coated targets can trigger numerous cellular effector functions including

phagocytosis, antibody-dependent cell-mediated cytotoxicity, respiratory burst, synthesis and release of cytokines and other inflammatory mediators (26). The IgA FcR expression increased in male calves due to the lactulose feeding. The availability of lactulose in the ileum may support the growth of the supplemented *E. faecium* and consequently could stimulate IgA FcR synthesis. Additionally, studies reveal that feeding lactulose is associated with increases in IgA secretion and IgA cells in GALT (27). Thus, FcRs provide a crucial link between the humoral and cellular arms of the immune system (28). The high-affinity FcγRI is not constitutively expressed. A high level on neutrophils and cytokines such as INF-γ need to be administered to up-regulate the receptor expression. However, we found no correlation between changes in IFN-γ production and IgA FcR expression in the ileum due to lactulose feeding (data not shown). In which way lactulose or prebiotics in general influenced the IgA FcR is still unclear.

The immunostimulatory effect of prebiotics has already been demonstrated through the proliferation and activation of immune cells, particularly CD4 CD8 T lymphocytes in peripheral blood and in the secondary lymphoid organ (18). A significant higher number of lymphocytes could be shown in the high lactulose L3 treatment. Other studies conducted with recognized prebiotic fibres have shown an alteration of the proportion of T cells (CD4 and CD8) and increased lymphocyte and leukocyte numbers in the GALT of canine (29) and in the peripheral blood of calves (30). Our results confirmed these studies though lactulose increased the lymphocytes in blood. Overall, the treatments did not affect the subpopulations of lymphocytes in male calves, whereas the CD4 T-lymphocytes of female calves in PP and mLN decreased and the CD8 T-lymphocytes in WBC increased. CD4 cells secrete a number of cytokines that are important in the activation of B cells, T cells and cells of the innate immune system, whereas CD8 cells play a role in immunological tolerance, such as the tolerance to foreign antigens encountered in the gut (31). Furthermore, the supplementation of 3% lactulose reduced the mRNA expression of cell surface receptor CD25 (α chain of the IL-2 receptor) in the mLN of male calves, which is expressed on activated T cells, B cells and monocytes. Formation of the high–affinity IL-2 receptor (expression of CD25) allows T cell proliferation and differentiation. Also the CD69 expression in spleen in the same feeding group indicating that lactulose is able to modulate the activation status of *in vivo* differentiated T cells. Low expression of CD69, a marker of leukocyte activation on CD8 cells could be attributed with a specific activation of CD3/CD8 T cells (32). The identification of the cellular subpopulations activated by different nonpathogenic bacterial strains was analyzed by Haller *et al.* (2000) (32) and only natural killer cells, which were activated by different nonpathogenic bacterial strains, are able to up-regulate activation markers.

Due to lactulose feeding the lymph follicles in the Peyers patches of the ileum revealed smaller sizes which were obvious sex-specific different (20). This could suggest a lower immunological activity throughout the lactulose rich feeding (20). The stabilization of the intestinal environment by the reduction of pathogen bacteria by lactulose could have lead to a reduced activation of the immune system. Because all calves were in the best of health, no possible decrease of infections could be analysed. The intention to scientifically prove the positive effects of lactulose in calf nutrition, as they are known from human nutrition, was only partly successful. Moreover, it need be noted, that high individual differences between the animals complicate the proof of the effectiveness of the lactulose concept.

CONCLUSION

The results indicate that the supplementation of lactulose to *E. faecium* fed calves has an immune modulatory effect on the lymphocyte content and on the composition of T cell subsets in different immune compartments. The supplementation of 1% lactulose induced more significantly immuno-modulation in female calves, whereas the supplementation of 3% lactulose affects the male calves in different immune

compartments. Further studies are needed to confirm these findings and better define the mechanisms for immunomodulation through lactulose, and the ultimate impact on health. There is convincing preliminary data to suggest that the feeding of lactulose can modulate immune parameters in GALT, secondary lymphoid tissues and peripheral circulation in pre-ruminant calves.

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FIGURES AND TABLES

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

Fig. 1. Single-run-specific efficiency-corrected relative expression. For normalization of each analyzed target gene in the presented study the mean expression of three reference genes, ß-Actin, GAPDH, and Ubiquitin was calculated. Further the efficiency of mentioned genes was included in the calculation model.

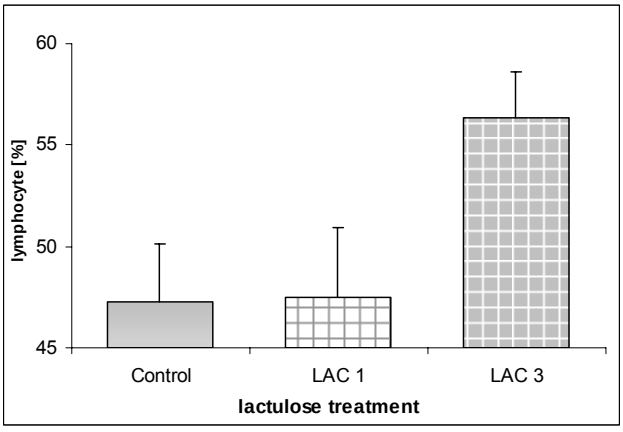

Fig. 2. Influence of lactulose feeding on lymphocytes in probiotic-fed calves.

Tab. 1. Primers used for quantitative one-step RT-PCR.

Identity	Sequence [5'-> 3']		Size [bp]	Accession No
ß-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		
Ubiquitin	FOR	AGATCCAGGATAAGGAAGGCAT	198	Z18245
	REV	GCTCCACCTCCAGGGTGAT		
IL-1ß	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-ß1	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		
IFN-γ	FOR	CTT GAA TGG CAG CTC TGA GAA AC	112	M29867
	REV	GGC CTC GAA AGA GAT TCT GAC		
IgA FcR (CD89)	FOR	GAC AAA CCC TTT CTC TCC ACC	180	AY247821
	REV	ACA GGA CCC AGA GTG AAG TC		
CD25	FOR	ATG GAG CCA AGC TTG CTG ATG T	171	NM_174358
	REV	TCT GCG GAA GCC TGT CTT GCA		
CD69	FOR	GTC ATT GAT TCT AAA GAG GAC ATG A	137	AF272828
	REV	AGG TTG AAC CAG TTG TTA AAT TCT		
CD4	FOR	GAT CGA GGT CTT GCC TTC AG	237	Multi
	REV	GAT CTG AGA CAT CCG TTC TGC		
CD8	FOR	ACT GTG TAT GGC AAG GAG GTG	127	XM585436
	REV	GGG TAT CCC AAT GAT CAT GCA G		

Tab. 2.: Diverse CD markers: CD4 and CD8 for the different population of lymphocytes, and the activation markers CD25 and CD69. Data are presented as relative expression (shown as x-fold regulation) in means of male and female calves ± SEM (n=7). 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 (+ $0.05<P<0.1$). The control groups have a constant level of 1 ± SEM.

			CD4			CD8			CD25			CD69		
tissue	**group**	**sex**	**x-fold reg.**	**SEM**	**P-value**	**x-fold reg.**	**SEM**	**P-value**	**x-fold reg.**	**SEM**	**P-value**	**x-fold reg.**	**SEM**	**P-value**
ileum	L1	male	1.0	0.28		1.67	0.49		1.62	0.57		1.78	0.42	
		female	0.40	0.06	*	0.42	0.11		0.89	0.43		0.65	0.12	
	L3	male	0.97	0.34		1.66	0.57		0.50	0.18		1.12	0.20	
		female	0.60	0.21		0.80	0.21		1.39	0.43		1.27	0.46	
mLN	L1	male	1.78	0.39	+	1.27	0.37		0.92	0.40		1.37	0.22	
		female	0.49	0.10	* *	0.96	0.26		1.58	0.52		1.20	0.28	
	L3	male	1.17	0.37		1.15	0.18		0.43	0.10	*	1.07	0.25	
		female	0.75	0.09		0.88	0.20		1.63	0.29		0.95	0.14	
spleen	L1	male	1.70	0.53		0.69	0.22		1.35	0.62		0.44	0.13	
		female	0.66	0.17		0.80	0.31		0.80	0.45		0.75	0.34	
	L3	male	0.90	0.17		1.56	0.43		1.09	0.31		0.35	0.11	*
		female	0.72	0.18		0.55	0.13		0.58	0.36		0.58	0.15	
WBC	L1	male	1.06	0.23		0.68	0.10		1.06	0.27		0.74	0.17	
		female	1.56	0.43		2.10	0.42	*	1.53	0.28		2.00	0.39	+
	L3	male	1.07	0.22		0.46	0.16		1.34	0.35		1.34	0.38	
		female	0.83	0.20		1.31	0.24		1.43	0.29		1.63	0.25	

Tab. 3.: Effect on the IgA FcR mRNA expression in the ileum and blood. Data are presented as relative expression (shown as x-fold regulation) in means of male and female calves ± SEM (n=7). 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 (+ $0.05<P<0.1$). The control groups have a constant level of 1 ± SEM.

			IgA FcR		
tissue	**group**	**sex**	**x-fold reg.**	**SEM**	**P-value**
ileum	L1	male	2.61	0.67	*
		female	1.29	0.28	
	L3	male	2.03	0.52	+
		female	1.75	0.49	
blood	L1	male	1.12	0.31	
		female	1.24	0.33	
	L3	male	2.19	0.73	
		female	1.43	0.45	