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Impact of prebiotic substances on gut health of livestock animals

Inulin, lactulose and Pinus massoniana pollen

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CONTENT

ABBREVIATIONS

AB-PAS	Alcian blue-periodic acid staining
ANOVA	analysis of variance
B. fragilis	*Bacteroides fragilis*
B. thetaiotaomicron	*Bacteroides thetaiotaomicron*
C. perfringens	*Clostridium perfringens*
C_T	cycle threshold
DNA	deoxyribonucleic acid
ECIS	electronic cell impedance sensing
E. coli	*Escherichia coli*
EDTA	ethylendiamintetraacetic acid
E. faecium	*Enterococcus faecium*
ESI	electro spray ionization
FOS	fructo-oligosaccharides
LC	liquid chromatography
mRNA	messenger RNA
MS	mass spectrometry
m/z	mass to charge ratio
n	number
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P. freudenreichii	*Propionibacterium freudenreichii*
P. densiflora	*Pinus densiflora*
P. massoniana	*Pinus massoniana*
qRT-PCR	quantitative RT-PCR
RIN	RNA integrity number
RNA	ribonucleic acid
RT	reverse transcription
S. aureus	*Staphylococcus aureus*
s.e.m.	standard error of means
ToF	time of flight

Names and abbreviations of genes investigated in the present study

In the following all genes that were investigated in the present study are listed with their full name, the abbreviation used in the text and tables. Additionally the accession number of the sequence that was used for primer design is given.

Cell culture experiment (*sus scrofa*):

Caspase 3 (Casp3)	NM_214131
Cyclin A2 (CCNA2)*	Multi*
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	AF017079
Histone H3.3A (H3F3A)	NM_213930
Interleukin 6 (IL6)	NM_214399
Interleukin 8 (IL8)	NM_213867
Transforming growth factor beta 1 (TGFB1)	NM_214015
Ubiquitin B (UBB)	NM_001105309

Feeding experiment (*bos taurus*):

antigen identified by monoclonal antibody Ki-67 (MKI67)	XM_590872
actin beta (ACTB)	NM_173979
BCL2-like 1 (BCL2L1)	NM_001077486
BCL2-associated X protein (BAX)	NM_173894
caspase 3 (CASP3)	NM_001077840
CD4 molecule (CD4)*	Multi*
CD8b molecule (CD8B)	NM_001105344
CD69 molecule (CD69)	NM_174014
epidermal growth factor receptor (EGFR)	XM_592211
glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	NM_001034034
G protein associated kinase (GAK)	NM_001046084
interleukin 1 beta (IL1B)	NM_174093
interleukin 2 receptor alpha (IL2RA)	NM_174358
interleukin 8 (IL8)	NM_173925
interleukin 10 (IL10)	NM_174088.1
interferon gamma (IFNG)	NM_174086
platelet/endothelial cell adhesion molecule (PECAM1)	NM_174571
peptidase (mitochondrial processing) alpha (PMPCA)	NM_001076964

ras-related protein Rab21 (RAB21)	XM_001249323
receptor for Fc fragment of IgA (FCAR)	NM_001012685
transforming growth factor beta 1 (TGFB1)	NM_001166068
tumor necrosis factor (TNF superfamily, member 2) (TNF)	NM_173966
ubiquitin (UBIQ)	Z18245
vacuolar protein sorting 4 homolog A (VPS4A)	NM_001046615

***** In some cases mRNA sequences of the gene of interest were not available for the species used in the study (*sus scrofa* or *bos taurus*). There primer design was performed on a consensus sequence from different species.

TABLES AND FIGURES

Tables:

Figures:

ABSTRACT

Prebiotic substances such as inulin or lactulose have been propagated to improve health of man and animal by stimulation of beneficial bacteria and their fermentation products in the intestine. Consequently prebiotics have been suggested as alternatives to antibiotics after the ban of antimicrobial growth promoters in food producing animals.

So the current work was performed to investigate effects of feeding 2% of either inulin or lactulose in milk replacer to male Holstein-Friesian calves. Therefore animal performance and haematologic traits were investigated. Additionally intestinal mucosa architecture and gut associated lymphoid tissue morphology were screened by means of histological analyses. Furthermore the mRNA expression patterns of selected genes involved in cell proliferation, apoptosis, inflammation and immune defence were studied.

After 20 weeks on the prebiotic diet inulin treated animals showed significantly higher daily weight gains than those fed with lactulose, control animals had intermediate values. Consumption of milk replacer was also reduced in the lactulose group. Villus height in jejunum ($P=0.07$) and ileum ($P<0.05$) showed inverse results to daily weight gains with an increase for lactulose and a decrease for inulin treated animals. These results were mirrored in the density of proliferative epithelial cells in the ileum that tended to be lower in the inulin and higher in the lactulose group ($P=0.08$). Both prebiotics tended to lower numbers of goblet cells in ileal villus tips ($P=0.07$). Haematology showed that both prebiotics also were able to significantly decrease thrombocyte counts ($P<0.01$) while only inulin was able to increase haemoglobin concentration ($P<0.005$) and haematocrit ($P<0.05$). The total leukocyte count was decreased by lactulose ($P<0.005$) and both inulin and lactulose tended to lower monocyte proportions ($P=0.073$). Expression patterns showed a decrease of pro-inflammatory TNF in jejunum of lactulose fed animals ($P<0.05$). In ileum expression of anti-inflammatory IL10 was increased by inulin ($P<0.05$). Both prebiotics were able to increase the endothelial cell junction molecule PECAM1 in colon ($P<0.05$). In the same gut segment expression of the lymphocyte activation marker IL2RA tended to be lowered by lactulose ($P=0.058$). In mesenteric lymphoid nodes expression of pro-inflammatory IL8 was significantly increased in the lactulose group ($P<0.05$). Genes involved in proliferation and apoptosis were affected in ileum. There expression of the

proliferation marker MKI67 was enhanced by inulin (P<0.01) while the apoptosis related BAX tended to be increased by both prebiotics (P=0.096).

All these results show a clear effect of the prebiotics inulin and lactulose on animal health, but effects differ between substances. While inulin improved animal performance and seemed to be able to influence iron absorption while simultaneously decreasing the absorptive surface in the small intestine, lactulose decreased animal performance in spite of improved intestinal architecture. Both treatments elicited beneficial effects on intestinal inflammatory signals but the mechanisms differed between increased expression of anti-inflammatory IL10 by inulin and decreased expression of pro-inflammatory markers by lactulose.

In a sub-study effects of a natural remedy in traditional Chinese medicine – pollen of *Pinus massoniana* – were investigated. This pollen has been reported to have prebiotic properties in animal studies. Nevertheless some effects of other pine pollen were attributed to their content of polyphenolic substances. The sub-study aimed to investigate potential beneficial effects of Masson pine pollen independent from its content of indigestible fibres. Therefore proliferation and mRNA expression patterns of porcine ileal cells after treatment with pine pollen extracts were studied by using ECIS technique and qRT-PCR respectively. Pollen extracts were used at a concentration equivalent to 1% unprocessed pollen in the cell culture medium.

Water and 50% ethanol extracts of Masson pine pollen significantly decreased cell proliferation (P<0.05 to P<0.01) while a 100% ethanol extract only transiently decreased cell growth (P<0.05 to P<0.001). Only the 50% ethanol extract was able to influence mRNA expression. Levels of pro-inflammatory IL6 and IL8 were up-regulated, while the proliferation-promoter CCNA2 was decreased (P<0.05). 80% methanol and hexane extracts did not show significant effects. Effective pollen extracts did have specific mass signals found by LC-ESI-ToF-MS but unfortunately none of these substances could be identified by the techniques available at the time of this study. Nevertheless Masson pine pollen extracts have been shown to have effects on cell proliferation and inflammation *in vitro* independent and partly opposing to those elicited by whole pollen *in vivo*.

ZUSAMMENFASSUNG

Es wurde bereits mehrfach gezeigt, dass Präbiotika wie etwa Inulin oder Laktulose einen positiven Einfluss auf die Gesundheit von Mensch und Tier haben können. Diese Einflüsse werden über die Stimulation probiotischer Bakterien und deren Metabolismus im Darm des Wirtes erzielt. Infolge dieser Erkenntnisse wurden Präbiotika bereits als mögliche Alternativen zu den mittlerweile nicht mehr zugelassenen antibiotischen Wachstumsförderern in der Haltung Lebensmittel liefernder Tiere gehandelt.

Die aktuelle Forschungsarbeit zielte darauf ab, mögliche positive Effekte der Anreicherung eines Milchaustauschers mit je 2% Inulin oder Laktulose auf Holstein-Friesian-Bullenkälber zu untersuchen. Dazu wurden Leistungsparameter und Blutwerte ebenso untersucht, wie der Aufbau der Darmschleimhaut und des darmassoziierten Immungewebes. Zusätzlich wurde nach Veränderungen im mRNA-Expressionsmuster ausgewählter Gene der Zellproliferation, der Apoptose, der Inflammation und der Immunabwehr gesucht.

Nach einer 20-wöchigen Fütterungsperiode zeigten Tiere der Inulin-Gruppe signifikant erhöhte tägliche Zunahmen im Vergleich zur Laktulose-Gruppe. Die Kontrolltiere lagen zwischen diesen beiden Gruppen. Gleichzeitig zeigten die Laktulose-Tiere eine verminderte Aufnahme des Milchaustauschers. Umgekehrt verhielt sich die Zottenlänge sowohl im Jejunum ($P=0.07$) als auch im Ileum ($P<0.05$), wo Laktulose zu längeren, Inulin jedoch zu kürzeren Villi führte. Dazu passend war die Dichte an proliferierenden Zellen im Ileum in der Inulin-Gruppe tendenziell verringert und in der Laktulose-Gruppe erhöht ($P=0.08$). Beide Präbiotika tendierten zu einer Verminderung der Becherzellen in ilealen Zottenspitzen ($P=0.07$). Hämatologische Untersuchungen zeigten verringerte Thrombozytenzahlen in beiden Präbiotikagruppen ($P<0.01$). Gleichzeitig führte die Inulin-Fütterung zu einer Erhöhung der Hämoglobinkonzentration ($P<0.005$) und des Hämatokrit-Wertes ($P<0.05$). Die Leukozytenkonzentration wurde durch Laktulose verringert ($P<0.005$) und beide Präbiotika tendierten zu einer Verringerung des Anteils der Monozyten ($P=0.073$). Die Genexpressionsmuster zeigten eine Abnahme des pro-inflammatorischen TNF im Jejunum der Tiere der Laktulose-Gruppe ($P<0.05$). Im Ileum war die Expression des anti-inflammatorischen IL10 durch Inulin erhöht ($P<0.05$) Beide Präbiotika führten zu einer Erhöhung des mRNA-Levels des

endothelialen Zell-Junction-Moleküls PECAM1 im Dickdarm ($P<0.05$). Im selben Darmabschnitt war in der Laktulose-Gruppe auch die Expression von IL2RA, einem Marker aktivierter Lymphozyten, tendenziell verringert ($P=0.058$). Laktulose-Fütterung führte in den mesenterialen Lymphknoten allerdings zu einer Erhöhung der Expression des pro-inflammatorischen IL8 ($P<0.05$). Proliferations- und Apoptose-Marker waren nur im Ileum betroffen. Dort führte Inulin zu einer erhöhten Bildung von MKI67 ($P<0.01$) während die Expression von BAX durch beide Präbiotika tendenziell erhöht war ($P=0.096$).

Zusammengefasst zeigen diese Ergebnisse einen klaren Effekt der Präbiotika Inulin und Laktulose auf die Tiergesundheit. Allerdings unterscheiden sich diese Effekte zwischen den beiden Substanzen. Während Inulin trotz einer verringerten Oberfläche im Dünndarm die Leistungsparameter verbesserte und nachgewiesen über verbesserte Blutwerte auch die Eisenabsorption positiv beeinflusst haben könnte, führte die Fütterung von Laktulose zu einer Verschlechterung der Tierleistung trotz verbesserter Darmarchitektur. Beide Behandlungen zeigten anti-inflammatorische Wirkungen im Darm, jedoch waren die Mechanismen mit einer Erhöhung anti-inflammatorischer (Inulin) bzw. einer Verringerung pro-inflammatorischer (Laktulose) Signale jeweils andere.

In einer kleineren Studie wurden die Effekte eines natürlichen Heilmittels der traditionellen Chinesischen Medizin – dem Pollen von *Pinus massoniana* – untersucht. Für diese Pollen wurden bereits typische Eigenschaften eines Präbiotikums nachgewiesen. Allerdings schrieben weitere Studien die Wirkungen der Pollen anderer Pinien deren Gehalt an Polyphenolen zu. In der vorliegenden Untersuchung sollte daher nach möglichen positiven Eigenschaften der Gelbkieferpollen gesucht werden, die von ihrem Gehalt an Faserstoffen unabhängig sind. Dazu wurden Kulturen einer ilealen Schweinezelllinie mit Extrakten der Pinienpollen in einer Konzentration behandelt, die dem Gehalt von 1% nativem Pollen im Zellkulturmedium entspricht. Mittels der ECIS-Technologie und der qRT-PCR wurden dann Zellproliferation und mRNA-Expression untersucht.

Wasser- und 50%-Ethanol-Extrakte verringerten die Zellproliferation signifikant ($P<0.05$ bis $P<0.01$) bis zum Ende des Experiments. Der 100% Ethanol-Extrakt schaffte dies nur über einen kurzen Zeitraum ($P<0.05$ bis $P<0.001$). Die Expression der ausgewählten Gene konnte nur durch den 50% Ethanol-Extrakt signifikant beeinflusst werden. Dieser führte zu einer erhöhten Expression der pro-

inflammatorischen Signalmoleküle IL6 und IL8 während CCNA2, ein Steuerprotein der Zellproliferation, signifikant herab geregelt wurde ($P<0.05$). Der 80% Methanol- und der Hexanextrakt hatten keine Einwirkungen auf die untersuchten Parameter. Eine Untersuchung aller Extrakte mittel LC-ESI-ToF-MS ergab für die wirksamen Pollenextrakte spezifische Massensignale, die in den unwirksamen fehlten. Allerdings war es nicht möglich diese Substanzen während der vorliegenden Studie zu identifizieren. Als Schlussfolgerung kann daher nur bestätigt werden, dass auch extrahierte Inhaltsstoffe von Pinienpollen Effekte auf Zellproliferation und Inflammation *in vitro* hatten, die z.T. den *in vivo* gewonnen Ergebnissen mit nativem Pollen widersprachen.

INTRODUCTION

Calf rearing and feed additives

During calf rearing digestive and other diseases and stress problems pose a severe economical risk especially during the first months of life. Some of these problems can arise in group pens by dissemination of pathogens between the animals or via nipple feeding (Waltner-Toews et al., 1986). Others are associated with stress factors during rearing such as diet, social isolation or small pens (Le Neindre, 1993) or during transportation and new instalment (Loerch and Fluharty et al., 1999). Problems associated with stress factors can be a dysfunction of the intestinal barrier with loss of ions and water or uptake of antigens. Translocation of pathogens may also be increased in stressed animals while numbers of beneficial bacteria may decrease. Consequences could be diarrhoea or intestinal inflammation (Söderholm and Perdue, 2001). Stress factors that strain the intestine can be met by treatments or intestinal stimulation to reduce loss of function (DeWitt and Kudsk, 1999). In recent years calf diseases such as diarrhoea were prevented or treated with therapeutic doses of antibiotics (Svensson et al., 2003). Antibiotic substances at low doses also are used solely as growth enhancers to increase animal performance. Especially the latter use led to problems with resistant bacteria and antibiotic residues in meat for human consumption (McEwen and Fedorka-Cray, 2002; van den Bogaard and Stobberingh, 2000). Consequently the European Union finally banned antimicrobial growth promoters from the year 2006 on (EG 183/2003) after a preceding ban of single substances. But a ban on these growth enhancers led to adverse effects on animal health and to a subsequent increase of the administration of therapeutic doses of antibiotics (Casewell et al., 2003). To overcome these problems some changes in animal rearing practices have to be made. These may include improved management practices, the use of vaccines or nutritional changes (McEwen and Fedorka-Cray, 2002). In an earlier study the acidification of milk replacer already has been found to decrease occurrence of and animal loss by diarrhoea (Fallon and Harte, 1983). During recent years new feed additives were tried such as addition of immuno-stimulants like colostrum or natural substances with direct or indirect influences on harmful bacteria such as herbal extracts, probiotics or prebiotics (Brambilla and De Filippis, 2005; Lallès et al., 2007). But benefits and risks of most of

these substances as feed additives for livestock animals still remain to be investigated.

The intestinal microbiota in calves

The intestine of higher organisms is populated by an enormous number of bacteria that belong to a multitude of different species (Collado and Sanz, 2007; Gibson and Roberfroid, 1995). These bacteria stand in close interaction with the host via effects on and reactions from the intestinal mucosa. Intestinal bacteria – whether considered beneficial or not – have more than once been shown to regulate gene expression patterns (Hooper et al., 2001; Lammers et al., 2002), cell proliferation (Scheppach et al., 1992 and 1995) or mucus production (Mack et al., 1999; Sharma et al., 1995). Influences can be either direct by bacterial signalling molecules or enterotoxins (Savidge et al., 2003) or indirect via fermentation products such as short chain fatty acids (Scheppach et al., 1992 and 1995). Substrates for these intestinal bacteria are principally dietary carbohydrates that were not digested and absorbed in the upper gastrointestinal tract (Cummings et al., 1989). Additionally substances provided by the host itself, such as mucus, can be fermented by intestinal bacteria (Macfarlane and Gibson, 1991). All in all the composition of this intestinal flora can massively influence the well-being of the host. Especially breakdown of the balance between harmful and beneficial bacteria and microorganisms, so called “dysbiosis”, can cause severe problems from digestive disorders to microbial infections causing diarrhoea or inflammatory bowel disease (Tamboli et al., 2004). Reports on investigation of intestinal flora in calves are scarce and these studies are mostly done only on faeces samples (Rada et al., 2006). But these data are not directly transferable to the whole intestinal tract since conditions change strongly within the gut segments (Macfarlane and Gibson, 1994). Collado and Sanz (2007) made an attempt to analyze mucosa associated gut microbiota in calves at different sites of the intestinal tract via culture techniques and fluorescence *in situ* hybridization (FISH) and flow cytometry. They found that total anaerobic bacteria counts were highest in rumen and decreasing to the colon. In duodenum highest proportions were found for strains of *Bacteroides,* and *Enterobacteriaceae* followed by *Staphylococcus, Propionibacterium* and *Enterococcus.* Least numbers were found for *Clostridium*, *Lactobacillus, Bifidobacterium* and yeasts*.* In colon all counts were lower except for *Bifidobacterium* and yeasts and the proportions were rather inversed with highest counts for

Bifidobacterium and lowest for *Bacteroides*. This domination of bifidobacteria in faeces was also found in another study (Rada et al., 2006). But in both studies only cultivatable bacteria were investigated or a small subset of bacteria species was searched for with FISH probes. This leaves the majority of intestinal bacteria species unclassified.

Concentrating on the bacteria that were detected in the studies mentioned above a division in predominantly harmless or even beneficial and predominantly harmful or even pathogenic bacteria can be made and was done e.g. by Gibson and Roberfroid (1995). This classification should be reviewed in the following.

Staphylococci, especially *S. aureus*, are pathogens that produce an array of very stable enterotoxins that can lead to symptoms of gastroenteritis. These superantigens stimulate non-specific T-cell activation and proliferation and lead to severe gastroenteritis symptoms (Balaban and Rasooly, 2000). For example staphylococcal enterotoxin B is known to impair intestinal barrier function e.g. by decreasing expression of tight junction proteins (Pérez-Bosque, 2006). Contaminated meat products can spread these toxins to humans (Balaban and Rasooly, 2000). Not surprisingly *Staphylococci* are considered as potential harmful bacteria species (Gibson and Roberfroid, 1995).

Similarly to *S. aureus Clostridia*, especially *C. perfringens*, are known to be enteric pathogens of human and domestic animals. Subtypes of *C. perfringens* are commonly found in the intestinal lumen also without disease and are facultative pathogens. Others are mostly associated with symptoms. These bacteria also secrete a variety of toxins causing a multitude of possible symptoms and diseases (Savidge et al., 2003; Songer, 1996). Many of these have been described for calves or adult cattle making *Clostridia* to a considerable cause of animal losses.

Enterobacteriaceae include some of the pathogens prevalently found in diarrhoea such as *E. coli*, *Proteus*, *Yersinia*, *Salmonella* or *Shigella* species, as well as *Klebsiella pneumoniae*, a pneumonia pathogen. Enteropathogenic *E. coli* or *Salmonella* infections are often found in calves of dairy farms especially when nipple fed (Waltner-Toews et al., 1986). Very early *Enterobacteriaceae* in calves and piglets were reported to be resistant to one or more commonly used antibiotics and were able to transfer these resistances to drug sensitive bacteria (Aden et al., 1969). So bacteria of this group are rather harmful, although there may be some probiotic

strains from *E. coli* such as *E. coli* strain Nissle, 1917 that is able to inhibit adhesion of pathogenic *E. coli* (Boudeau et al., 2003).

Enterococci are wide spread intestinal bacteria that are able to induce diseases especially outside the intestine such as bacteremia, endocarditis or infections of the central nervous system. An increasing problem is their ability to acquire resistances against several antibiotics simultaneously making them quite dangerous nosocomial bacteria (Murray, 1990). Nevertheless within the gut strains of *E. faecium* have been found to have probiotic character and preventive activity against *Salmonella* infections (Audisio et al., 2000). But in recent years some concerns have been mentioned that probiotic *E. faecium* could be a recipient for antibiotic resistance gene clusters (Lund, 2001). A certain beneficial activity within the gut remains to be expected.

Bacteroides have been found to be predominantly harmless and even beneficial commensals or rather symbionts within the intestine. These bacteria are able to ferment a wide range of polysaccharides and provide the host with volatile fatty acids. *B. thetaiotaomicron* even has been reported to cross-talk with epithelial cells resulting in an increase of expression of different genes including a bactericidal lectin. Additionally it is involved in development of gut associated lymphoid tissue and immune response. On the other hand *Bacteroides* also have a pathogenic character. *B. fragilis* has potent virulence factors and leads to severe microbial infections and abscess formation especially when it is able to leave the intestine (reviewed by Wexler, 2007). Nevertheless the beneficial character seems to outweigh possible risks especially when considering the predominance of *Bacteroides* in the gut.

When talking about probiotics *Propionibacteria* are seldom mentioned. But there is also increasing evidence for a probiotic activity of e.g. *P. freudenreichii.* These bacteria are able to produce propionic acid and bacteriocins and they provide the host with vitamin B_{12}. Additionally they seem to induce growth of other beneficial bacteria in the intestine (Mantere-Alhonen, 1995). In combination with other probiotics *P. freudenreichii* has been shown to inhibit adhesion of human pathogens to intestinal mucus (Collado et al., 2006).

Bacteria from the genus *Lactobacillus* are commonly considered as probiotic bacteria and many strains are used for fermented milk products. As the name is suggesting their main fermentation products include lactic acid (Hadadji and Bensoltane, 2006) leading to a decreased intestinal pH value and simultaneously decreased ammonia

concentrations (Benno et al., 1996). *Lactobacilli* have been reported to have a multitude of beneficial effects on the host including reduction of rotaviral or antibiotic associated diarrhoea, cancer-preventive actions, immune modulation, lowering of low density lipoprotein-cholesterol or improvement of symptoms of inflammatory bowel disease (Ouwehand et al., 2002). Additionally they have been found to inhibit adherence of pathogenic *E. coli* to intestinal mucins (Mack et al., 1999) while there are also reports that intestinal counts of beneficial bifidobacteria are increased by certain *Lactobacillus* strains (Benno et al., 1996). Thus their presence in normal microflora can be considered as beneficial for the host.

Bifidobacteria are probably the most important beneficial bacteria within the gut especially of infants and young animals. Presence or absence of these bacteria has been shown to be indirectly proportional to absence or presence of harmful bacteria such as *Clostridia* species or *E. coli* (Rada et al., 2006). Similar to *Lactobacilli* these bacteria have been reported to produce strong organic acids such as lactic acid or acetat (Gibson and Roberfroid, 1995; Hadadji and Bensoltane, 2006). Organic acids in the gut have been repeatedly reported to prevent or combat infection with *Salmonella* (Van Immerseel et al., 2006). A further defence against malignant cells or pathogens may be the immune modulatory properties of these bacteria (Gibson and Roberfroid, 1995; Mitsuoka, 1990) or their ability to produce bacteriocins and similar antimicrobial substances (Collado et al., 2005). Additionally cross-feeding of other bacteria by fermentation products of bifidobacteria can also increase formation of butyrate (Van Immerseel et al., 2006) which has been reported to be a main energy source of colonocytes (Scheppach et al., 1992). Acidification of the gut lumen also increases proportion of ammonium ions and thus decreases the amount of non-protonated and toxic ammonium in the blood. Furthermore bifidobacteria produce vitamins and digestive enzymes that are important for the host (Gibson and Roberfroid, 1995).

Health promoting effects of prebiotic substances

Prebiotics are defined as non-digestible substances that beneficially affect a host by modulation of its intestinal flora by stimulation growth or activity of selected microorganism. These substances need to reach lower parts of the gastrointestinal tract and to be a selective substrate for a limited number of beneficial commensals. These bacteria have to be stimulated to grow and to increase metabolic activity. The

intestinal microflora has to be altered to a more beneficial equilibrium and all these changes have to lead to health promoting effects for the host (Gibson and Roberfroid, 1995). Some indigestible oligosaccharides commonly viewed as prebiotics are fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides, xylo-oligosaccharides, soya oligosaccharides or isomalto-oligosaccharides. Additionally the disaccharide lactulose is also considered a prebiotic. But most of the studies are performed on the closely related fructans inulin and FOS (Macfarlane and Cummings, 1999). From all these substances mentioned above the present study is concentrating on inulin and lactulose.

Inulin is a natural occurring ingredient of food since it is a content of a wide range of plants such as vegetables and cereals of the western diet (Van Loo et al., 1995). Its chemical structure is β-(2-1)-linked FOS with 2 to 60 sugar units (figure 1). A starting glucose moiety may or may not be present. In the intestine of man and animal inulin is selectively fermented by a small number of bacteria and leads to a shift in the microbial flora towards more bifidobacteria and less pathogens like *E. coli*. This shift is commonly regarded beneficial for host health (Flickinger et al., 2003; Gibson et al., 1995). Inulin-type fructans have also been reported to show anti-carcinogenic properties (Hughes and Rowland, 2001; Femia et al., 2002) and to stimulate immune functions (Schley and Field, 2002). Furthermore lowering effects on serum lipid levels have been found that were based on influences on lipid metabolism (Delzenne and Kok, 1999; Delzenne et al., 2002). In livestock animals, including calves, inulin-type fructans have been shown to enhance performance (Van Loo, 2007).

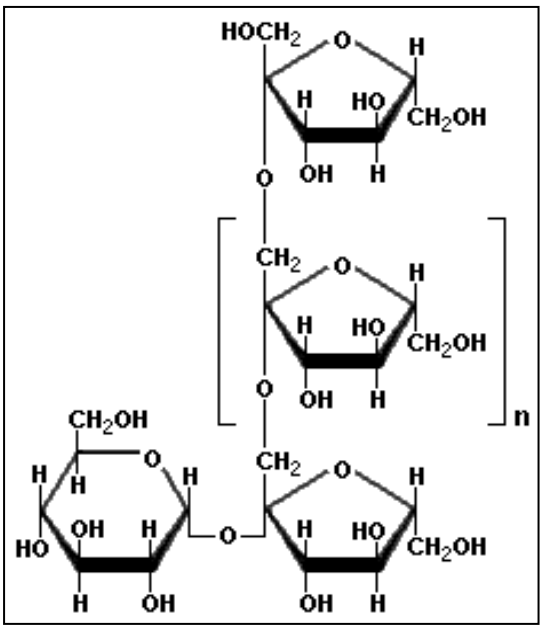

Figure 1: *Chemical structure of inulin*

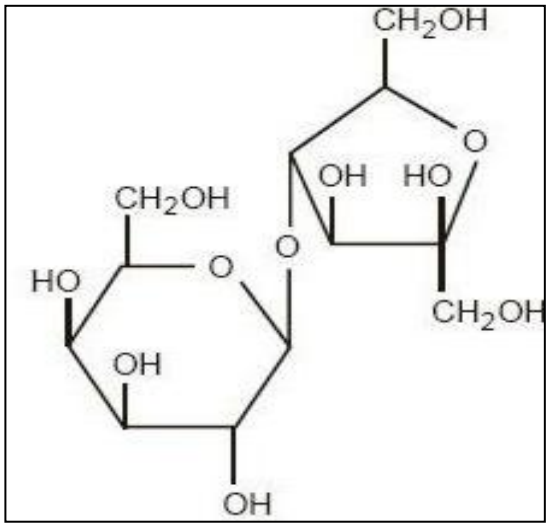

Figure 2: *Chemical structure of lactulose*

In contrast to inulin lactulose does not occur naturally. This disaccharide (figure 2, 4-O-β-D-galgactopyranosyl-D-fructose) is semi-synthetically produced from lactose and typically is formed in heat treated milk by a non-catalyzed isomerization. Although not a natural product it has been found to have prebiotic properties (Schumann, 2002). Lactulose mainly is fermented by lactobacilli and bifidobacteria, but also by other bacteria species such as *C. perfringens*, *E. coli* or *Bacteroides* species (Mitsuoka et al., 1987). Lactulose is commonly used to treat constipation especially in elderly people (Attar et al., 1999). But it also has been used in the treatment of hepatic encephalopathy. There its main effective action beside inducing diarrhoea and changing the intestinal flora was a reduction of blood ammonia levels causative for the symptoms of the disease (Bircher et al., 1966). But similar to inulin also beneficial effects on carcinogenesis, the immune system or animal performance have been reported (Fleige et al., 2007a; Schumann, 2002; Verdonk et al., 2005). For example calves that were given lactulose and colostrum had significantly higher amounts of anti-bacterial C-reactive protein in blood serum than control animals probably due to a selective stimulation of passive protein transport from gut lumen into the blood (Schroedl et al., 2003). Also in a feeding experiment with periparturient sows and their piglets beneficial effects of lactulose on intestinal flora, immune system and offspring development (Krueger et al., 2002).

Additionally to the above mentioned indigestible saccharides some other natural products have been proposed to have prebiotic character. One of these may be the pollen of the Chinese yellow pine, *Pinus massoniana*. This pollen has been used in traditional Chinese medicine for a long time for its health supporting effect. Especially treatment of disorders of the digestive system has been an important field of application (Choi, 2007). In modern times pine pollen is also used in the food and cosmetic industry with massive advertisement of its claimed beneficial effects. But up until now only a small number of studies on the beneficial effects of pine pollen have been conducted. The application of pine pollen to treat constipation and other intestinal diseases suggests a possible prebiotic functionality. Truly pine pollen has been found to deliver only little metabolizable energy and to contain mostly cell wall components (Zhao et al., 1996) that may serve as substrates for bacterial growth. Polysaccharides from pine pollen have been reported to be composed of e.g. D-xylose (xylo-oligosaccharides) (Bouveng, 1963). Such oligosaccharides have been proposed to possibly have prebiotic character (Macfarlane and Cummings, 1999).

These probably prebiotic compounds may be the cause for lowered counts of the pathogens *Proteus mirabilis* or *E. coli* in the intestine of rats fed Masson pine pollen (Zhao et al., 1996). Additionally this pollen has been found to modify mRNA expression levels of inflammatory or cell cycle associated genes in colon of piglets, also effects typically associated with prebiotic substances (Schedle et al., 2008). However regarding pollen substances not all results reported can be or were explained by a prebiotic character. Some studies with *P. densiflora* pollen extracts found anti-inflammatory, anti-nociceptive and anti-oxidative effects that were attributed to the content of polyphenolic substances (Choi et al., 2007; Lee et al., 2008). These substances have been found in pine pollen before (Strohl and Seikel, 1964) and are known for their anti-oxidative, anti-carcinogenic and immune stimulatory effects (Lambert et al., 2005; Sehm et al., 2006). So, *P. massoniana* pollen may have additional effects on animal health beside a prebiotic activity.

AIMS OF THE STUDY

Prebiotics are of considerable interest in animal husbandry but up until now only little is known on their effects in livestock animals. For *P. massioniana* pollen a certain prebiotic action has already been shown in pigs. But evidences exist, that additional effects independent from their content of indigestible fibre may play a role in their activities. These effects should be revealed during cell culture experiments with pollen extracts and porcine ileal cells. Therefore changes of cell proliferation and mRNA expression patterns were investigated and an identification of the causative substances was attempted.

Two substances, inulin and lactulose, that have long been known to have prebiotic character in humans and model animals shall now be investigated for their potential beneficial effects on calf rearing. For inulin only little information is available concentrating nearly totally on intestinal flora and animal performance (figure 3). Some additional insight on lactulose effects on calves has been achieved by only one other study. These findings are summarized in figure 4. This knowledge had to be verified and deepened during the present research work. Namely effects on animal health needed to be investigated via observation of possible changes in animal performance, intestinal architecture, haematologic traits and mRNA expression patterns of selected genes including inflammatory and immune modulatory genes.

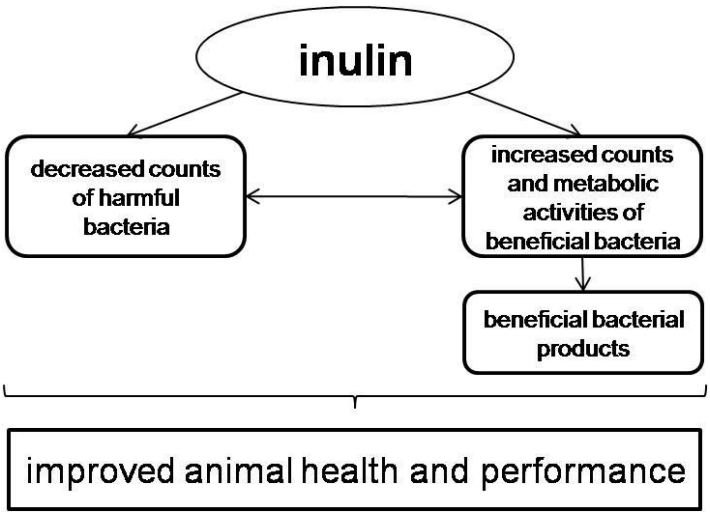

Figure 3: *Knowledge on effects of inulin on calf health and performance before the present research work*

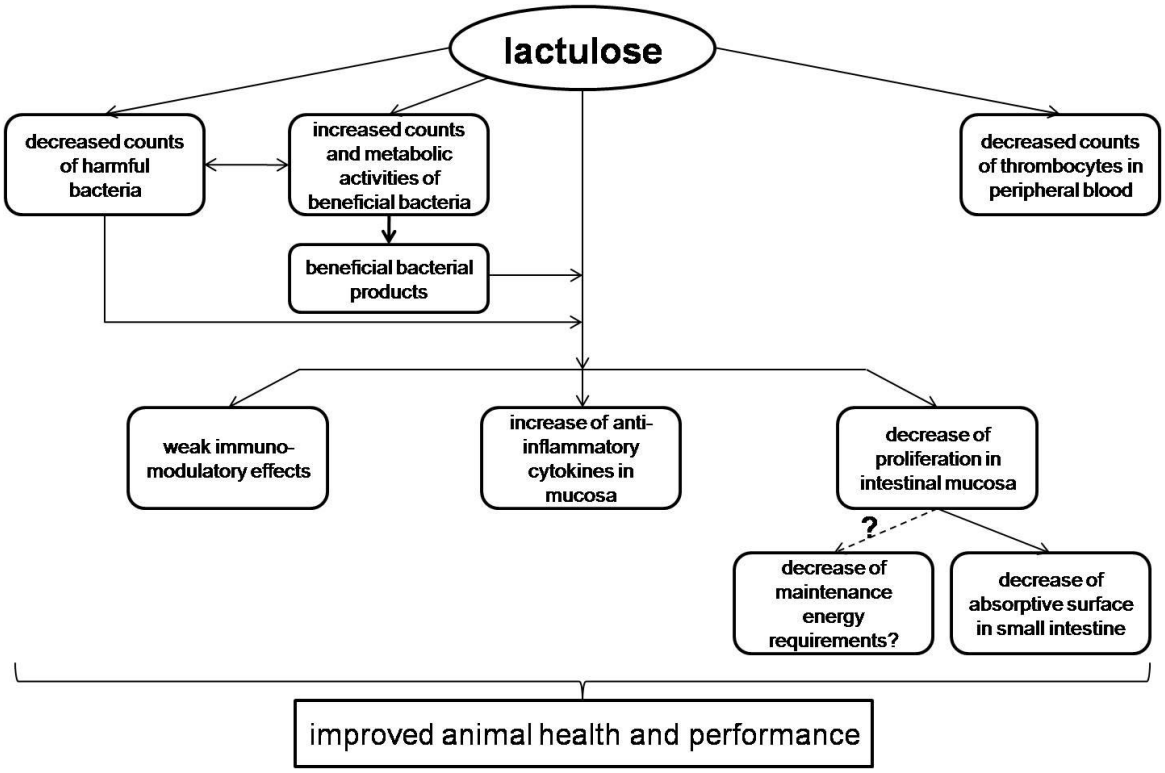

Figure 4: *Knowledge on effects of lactulose on calf health and performance before the present research work*

MATERIAL AND METHODS

in vitro investigation: effects of pine pollen extracts on porcine ileal cell culture

Pollen compound extraction

To test for effects of *P. massoniana* pollen beside its postulated prebiotic properties extracts had to be made to get preparations of different soluble compounds independent from insoluble lignin or cellulose components. To better release internal compounds the pollen was broken with a ball mill (Retsch GmbH & Co. KG, Haan) as was described previously (Masanetz et al., 2009). Before grinding the pollen was shock frozen with liquid nitrogen to prevent thermal damage to potentially heat sensitive compounds. In the following 10 g of ground Masson pine pollen were extracted with 60 ml of one of the following solvents: water, 50% ethanol, 100% ethanol or a mixture of hexane and 80% methanol (v/v=1:1). For extraction pollen and solvents were mixed in glass bottles with silicone covered lids and shaken for 24 h at room temperature. To recover supernatants the mixtures were then centrifuged to pellet pollen debris. Hexane and 80% methanol formed two distinct phases which were further processed individually. Extracts were dried using either vacuum centrifugation (RC 10.22, Jouan, Tring, Great Britain) for organic solvents or lyophilization (Christ Alpha 1-4, Martin Christ GmbH, Osterode, Germany) for water extracts. Remaining residues were weighed and were then dissolved in sufficient amounts of phosphate buffered saline (PBS) containing 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich, Steinheim, Germany) to enhance solubility of lipophilic substances. Extracts were sterile filtered (Minisart 0.2 µm, Sartorius AG, Göttingen, Germany) and stored at -20°C. For control treatments extracts were produced with the same solvents but without addition of pollen. These extracts were made to correct for substances that may be present in solvents or containers. Concentrations of resolved extracts were calculated to amount of unprocessed pollen per volume.

Determination of cell proliferation

Cell proliferation of the porcine ileal cell line IPI-21 was investigated with an electric cell-substrate impedance sensing device (ECIS Model 1600, Applied Biophysics, Troy, New York). Thereby cell attachment and growth were measured via changes in impedance in a cell culture well under a weak electrical current. This allowed a highly

reproducible real-time monitoring of the development of a cell culture under different conditions. Cells were cultured at a density of 2.0 x 10^4 cells/well in 300 µl medium in 8-well arrays (ECIS Cultureware 8W10E, Applied Biophysics) fitted with 10 electrodes per well. Extracts were added to a final concentration that was equivalent to the addition of 1% (w/v) whole pollen to the medium. Control treatments were performed simultaneously with equal amounts of corresponding control extracts. Impedance was measured every three minutes at 30 kHz. All values for each well were normalized to the first obtained immediately after addition of cells and before they were attached.

Total RNA extraction and expression analysis

After 48 h of cultivation total RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Cells were washed twice with cold PBS and then were lysed directly in the culture well using the lysis buffer of the kit. After purification RNA concentrations were measured with a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) and diluted to a stock solution of 100 ng/µl and subsequently to a working solution of 10 ng/µl. RNA quality was checked to be sufficient with a Bioanalyzer 2100 (Agilent, Palo Alto, CA).
Quantitative reverse transcription PCR (qRT-PCR) was done with a Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) and the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). Specific primer sets for porcine sequences obtained via the NCBI homepage (http://www.ncbi.nlm.nih.gov/) were designed using primer3 v. 0.4.0 (http://frodo.wi.mit.edu/). If possible they were chosen to span exon-exon boundaries on the mRNA sequence to minimize amplification of genomic DNA. Acceptable primer sequences were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Identities of the RT-PCR products were verified by sequencing (Sequencing Service, Ludwig Maximilian Universität München, Germany). The run protocol was the same for all primer pairs: reverse transcription (10 min at 50°C), denaturation (5 min at 95°C) and an amplification and quantification step consisting of 45 cycles (15 s at 95°C, 30 s at 60°C, 20 s at 68°C). Primer sequences and product sizes are given in table 1. For normalization housekeeping genes H3F3A, UBB and GAPDH were chosen. At least two of them were stably expressed in every control-treatment pair and those were used for statistical analysis.

Table 1: *Primer pairs and PCR product sizes for qRT-PCR in the cell culture experiment*

gene	primer sequence		size
reference genes			
H3F3A	for	ACTGGCTACAAAAGCCGCTC	232 bp
	rev	ACTTGCCTCCTGCAAAGCAC	
UBB	for	AGATCCAGGATAAGGAAGGCAT	198 bp
	rev	GCTCCACCTCCAGGGTGAT	
GAPDH	for	AGCAATGCCTCCTGTACCAC	187 bp
	rev	AAGCAGGGATGATGTTCTGG	
genes of interest			
CASP3	for	TGTGTGCTTCTAAGCCATGG	158 bp
	rev	AGTTCTGTGCCTCGGCAG	
CCNA2	for	GCAGCAGCCTTTCATTTAGC	116 bp
	rev	TGAAGGTCCAGGAGACAAGG	
TGFB1	for	TACTACGCCAAGGAGGTCAC	155 bp
	rev	TCTGCCCGAGAGAGCAATACA	
IL6	for	AAGGTGATGCCACCTCAGAC	151 bp
	rev	TCTGCCAGTACCTCCTTGCT	
IL8	for	GGCAGTTTTCCTGCTTTCTGC	153 bp
	rev	CAGTGGGGTCCACTCTCAAT	

Pollen compound analysis

A high performance liquid chromatography (LC) system (1200 Series LC) was coupled by electrospray ionization (ESI) to a time-of-flight mass spectrometry (ToF-MS; 6210 ToF LC/MS, all Agilent, Santa Clara, USA). This LC-ESI-ToF-MS was applied to possibly achieve information about compounds of the different extracts.

For analysis Masson pine pollen extracts were further diluted to a final concentration of 0.2% (2-hydroxypropyl)-β-cyclodextrin. LC was performed with a Prontosil 120-3-C18 reversed-phase column (Bischoff Chromatography, Leonberg, Germany) and a mixture of methanol and 10 mM ammonium acetate solution (pH 7.4) for separation. Methanol concentrations were increasing from 10% to 90% (v/v) within 8 min. The eluate was vaporized via ESI technology and the screening analysis was done by

simultaneously detecting positively and negatively charged ions with a high-resolution and high-accuracy ToF-MS. Further details can be found in a recently published study using a similar analytical set-up (Grosse and Letzel, 2007). Data analysis was done with the software Mass Profiler (Agilent). Signals were assumed as individual substances when a difference in retention time was greater than 0.2 min and/or molecular weight differed more than 1 Da.

Pine pollen extracts were directly compared to corresponding control extracts to eliminate signals originating in the extraction procedure. Figure 5 shows an exemplary comparison of the total ion chromatograms of a pollen and its corresponding control extract. Signals that are present in both solutions hint to residues that remained after evaporation of the extraction liquid or that are present in the PBS/(2-hydroxypropyl)-β-cyclodextrin solution. Especially the massive peak between 5.5 and 9 min is expected to represent (2-hydroxypropyl)-β-cyclodextrin since it was absent in a test extraction without addition of this solubilizer (data not shown).

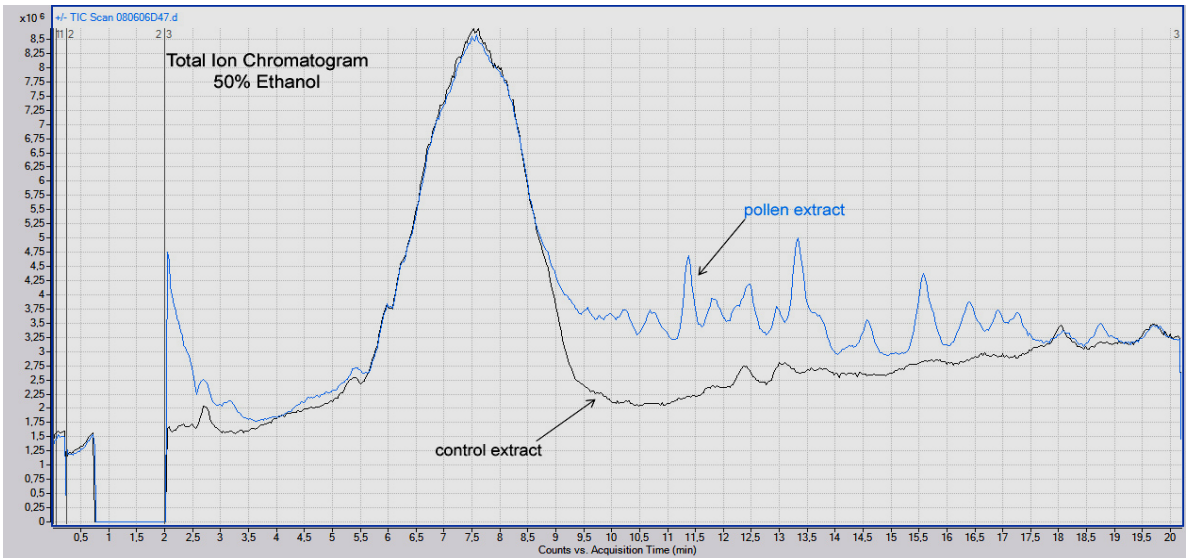

Figure 5: *Exemplary comparison of the total ion chromatogram of a Masson pine pollen (blue) and the corresponding control extract (black, 50% ethanol extracts) to identify pollen specific compounds.*

To determine molecular weights of the molecules observed m/z spectra were analyzed. Mass signals that were only present in the pollen extracts were considered for further investigation. These signals in turn were compared between the different pine pollen extracts to determine signals specific for single extracts. For an example for this comparison see figure 6. These signals were compared to a library of

phenolic compounds found in red wine that has been established at the laboratory during a previous work.

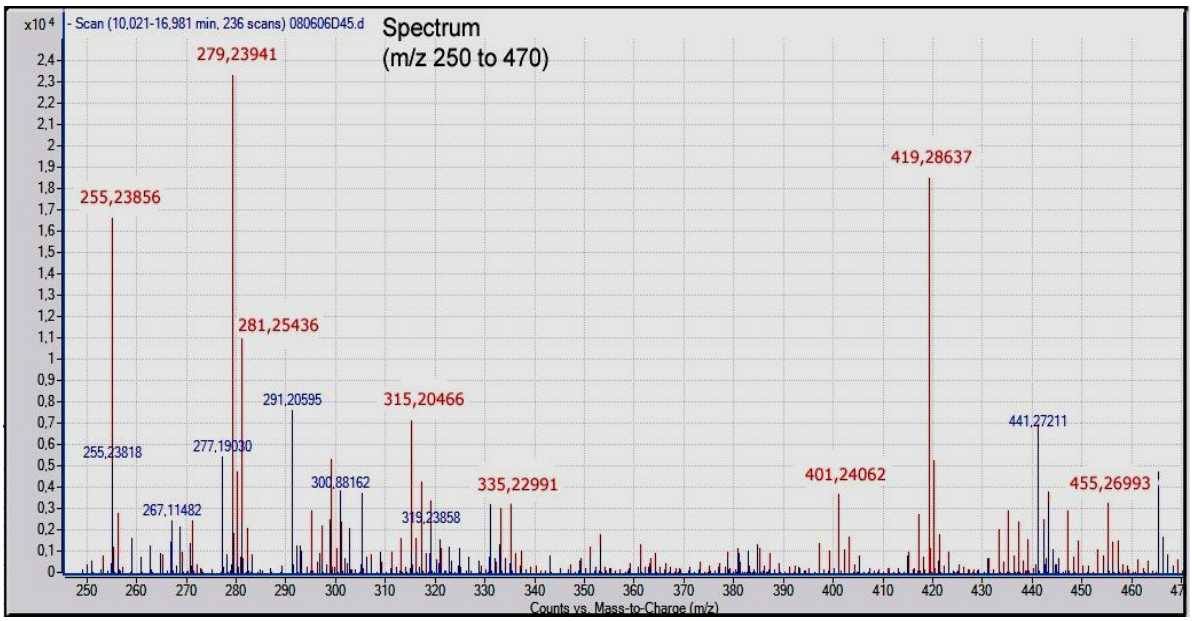

Figure 6: *Exemplary comparison of the m/z spectra of the water (blue) and the 50% ethanol (red) Masson pine pollen extract to find pollen compounds with different solubility that may be present in effective and absent in non-effective extracts.*

Statistical analysis

Cell proliferation data is shown as mean normalized impedance values + standard error of the mean (s.e.m.) with n=4. Statistical analysis of cell proliferation results was done using SigmaStat 3.0 (SPSS Inc., Chicago, IL). A Student's t-test was performed for each time point giving an overview of statistical relevancies over the whole period of cell growth. Significant differences were declared when over a period nearly all values did reach significance levels at $P<0.05$. Relative quantification and statistical analysis of gene expression results were performed with REST 2008 V.2.0.1 (Technische Universität München, Corbett Life Science). In both cases four controls were compared to four treated cell culture wells. Results are given as mean fold changes compared to the expression levels in control treated cells. A significant difference between pollen and control extract treatment was declared at values of $P<0.05$.

in vivo investigation: effects of inulin and lactulose on preruminant calves

Animals, husbandry, feeding and experimental procedures

42 Holstein-Friesian bull calves were purchased from the Viehzentrum Waldkraiburg GmbH. These animals were subdivided into three experimental feeding groups, namely control, inulin and lactulose fed. All three groups had balanced weight (52.9±6.2 kg) and age (22±5 d). The animals were housed at the experimental station Karolinenfeld (Bayerische Landesanstalt für Landwirtschaft – LfL, Institut für Tierernährung und Futterwirtschaft) (Masanetz et al., 2010a/b).

Calves of the control group were fed the milk replacer Milkibeef Top (Milkivit, Trouw Nutrition, Burgheim, Germany). The two experimental feeding groups were fed the same milk replacer iso-energetically and iso-nitrogenically enriched with either 2% inulin (Beneo ST, Orafti, Tienen, Belgium) or 2% lactulose (Lactusat, Milei GmbH, Leutkirch, Germany). Compositions of all three diets are given in table 2. Calves were fed restricted rations individually by transponder automatic feeders (Förster Technik, Engen, Germany). Collected volumes were automatically recorded. During the experimental period the milk replacer concentration was rising from 125 g/L to 200 g/L with daily intake allowances rising from 6 L to 16 L. Calves had free group access to fresh drinking water and up to 300 g hay per day and animal. The animals were housed on straw, so a further uptake of roughage could not be excluded. The animals were slaughtered after 20 weeks.

Tissue sampling

At slaughtering the gastrointestinal tract was removed from the carcass immediately after the death. For gene expression studies pieces of central parts of jejunum, ileum and colon, of mesenteric lymph nodes and from the tip of the spleen were collected. Gut samples were washed twice in physiological salt solution. Tissue pieces were then immediately flash frozen in liquid nitrogen to avoid degradation of RNA. Until RNA extraction samples were stored at -80°C.

For histological analysis of the gut mucosal architecture cross-sections of 0.5 to 1 cm of centre parts of jejunum, ileum and colon were collected and washed in physiological NaCl solution. Also for histological investigation of lymph follicles mesenteric lymphoid nodes were taken. Tissue samples were placed in neutral buffered 3.7% formalin (Carl Roth GmbH, Karlsruhe, Germany) for 24 h. At the

Landesuntersuchungsamt (LGL, Oberschleißheim) samples where embedded in paraffin.

Table 2: *Ingredients and analysis of nutrient and energy content of the diets (energy content of the milk replacer was estimated with the program Zifo (LfL, 2005))*

	control	**inulin**	**lactulose**	**hay**	**straw**
ingredients (%)					
50% fat concentrate*	38.3	38.3	38.5		
skimmed milk	50.2	50.2	50.2		
pregelatinised wheat starch	4.4	2.8	3.5		
whey protein concentrate	4.1	3.5	-		
Beneo ST	-	2.2	-		
Lactusat	-	-	4.8		
vitamin/mineral/amino acid mix	2.0	2.0	2.0		
soy bean oil (+20% emulsifier)	0.985	0.985	0.985		
aroma	0.015	0.015	0.015		
analysis of nutrient and energy content					
dry matter (DM, g/kg)	963	959	960	880	892
crude ash (g/kg DM)	73	73	70	39	32
crude protein (g/kg DM)	225	229	230	112	35
ether extracts (g/kg DM)	210	208	211	15	13
crude fibre (g/kg DM)	6	2	4	323	460
energy (MJ/kg DM)	16.9	16.9	17.0	9.5	6.9

* 50% whey powder, 50% coconut/palm oil

Blood sampling

For haematology blood samples (15 mL) were collected into 8% EDTA and serum vacuum tubes (Greiner BioOne GmbH, Frickenhausen, Germany). The first was taken ten weeks after the beginning of the feeding experiment, the second during the slaughtering process after 20 weeks of feeding. Additional blood smears were also made. Examination was performed by a veterinary laboratory (Vetmed Labor, Unterhaching, Germany) according to their internal standards. Haemoglobin concentration and haematocrit were determined as well as thrombocyte, erythrocyte and total leukocyte numbers. The blood smear was used to obtain a differentiated

white blood cell count. 10 mL of slaughtering blood samples were also transferred to paxGene Blood RNA tubes (BD, Heidelberg, Germany) for RNA extraction. These tubes were stored at -20°C until extraction.

Histological analyses

To examine paraffin embedded tissue sections of 4-6 µm were cut (Microtom LEICA RM2145, Leica, Wetzlar, Germany), mounted on glass slides, deparaffinized and rehydrated before further treatment. All microscopic analyses were done randomized by one person without knowledge of treatment groups.

Sections were stained using either haematoxylin (Carl Roth GmbH, Karlsruhe, Germany) and eosin yellowish solution (Fluka-Chemie AG, Buchs, Switzerland) or the Alcian blue/periodic acid Schiff's reagent (AB-PAS) staining method. The latter is mainly used to detect goblet cells, but all other basic measurements can also be done on such stained tissues. Briefly sections pre-treated with 3% acetic acid were treated with 1% Alcian blue in 3% acetic acid and subsequently with 0.5% periodic acid. Tissues were then treated with Schiff's reagent (all chemicals from Carl Roth GmbH, Karlsruhe, Germany) and counterstained with haematoxylin.

Histomorphometry was done on AB-PAS or haematoxylin/eosin stained sections. Measurement techniques were adapted from Sehm *et al.* (2006). Briefly villus width and length from *lamina muscularis* to tip were measured in sections of jejunum and ileum. Crypt depth from *lamina muscularis* to the crypt mouth and distance between crypts were examined in sections of the colon. Figure 7 gives an overview of conducted measurements on the intestinal mucosa. All were done on three well defined villi or crypts of one section. In mesenteric lymphoid nodes and in ileal Peyer's patches the number of lymph follicles in a defined area and areas of these follicles were measured.

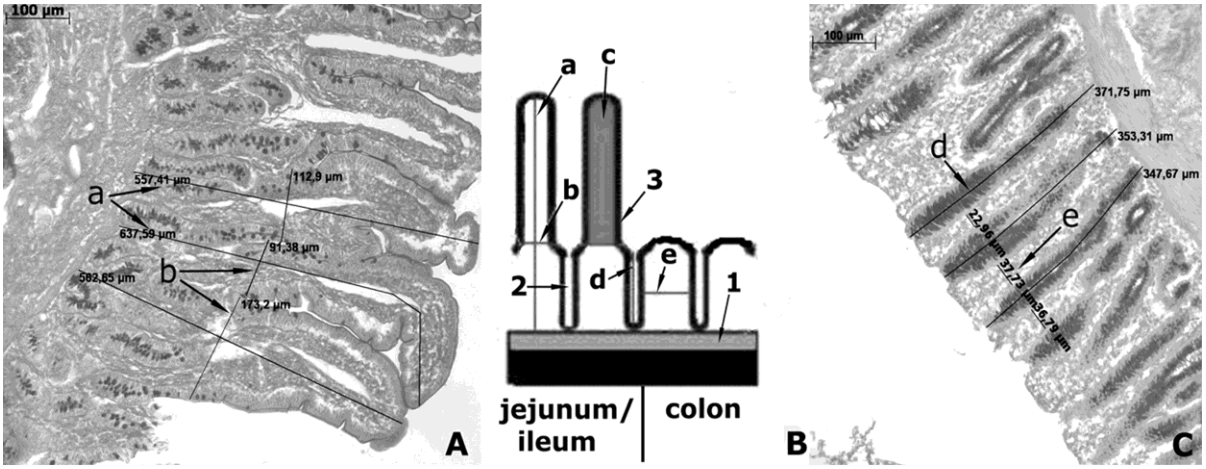

Figure 7: *Measurements on gut mucosal morphology in the large and small intestine: (B) shows a schematic overview over conducted measurements in the intestine. Examples for actual data acquisition are given for jejunum (A) and colon (C). (1)* lamina muscularis mucosae*, (2) Lieberkühn crypt, (3) villus; small intestine: (a) villus length, (b) villus width, (c) villus tip; large intestine (no villi existent): (d) crypt depth, (e) distance between crypts*

Histochemistry, namely the AB-PAS staining method, was used to detect goblet cells in jejunum and ileum. AB-PAS positive cells were counted individually for crypts and villi of jejunum and ileum. Goblet cell numbers in the colon were too great for quantification. Goblet cell density was then calculated as number of AB-PAS positive cells per length of villus or crypt outline (figure 8).

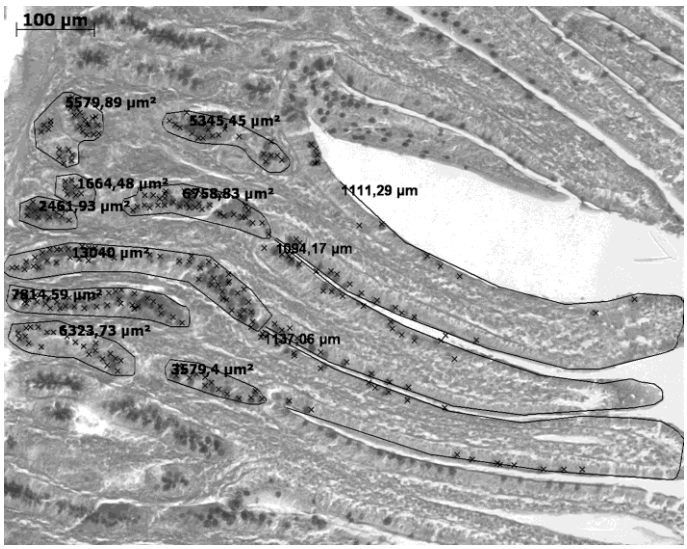

Figure 8 *Quantitative analysis of goblet cells: Goblet cell density was determined as number of goblet cells (x) per length of villus or crypt outline. The same counting technique was used to quantify proliferative cells.*

Detection of proliferative cells was done by immunohistochemistry on six randomly selected samples of each treatment group. To stain proliferative cells the antibody MIB-1 (Dako cytomation, Glostrup, Denmark) directed against the proliferation marker MKI67 was used. For antigen accessibility sections were incubated in boiling 0.01 M citrate buffer (pH 6). Between incubation steps slides were washed in PBS with 0.05% Tween. To block endogenous peroxidase activity the sections were treated with 1% hydrogen peroxide. Unspecific antigen binding was prevented by blocking with 10% goat serum (Dako cytomation). Incubation with the primary antibody MIB-1 (1:50 in PBS) was done at 4°C over night. The next day sections were incubated with HRP-labelled polyclonal goat anti-mouse antibody (1:50 in PBS, Dako cytomation). Binding of secondary antibody was visualized by reduction of 3,3'-diaminobenzidine solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in the presence of 0.01% hydrogen peroxide. Tissue was then counterstained with haematoxylin. Quantification of MIB-1 positive cells was done similarly to AB-PAS positive cells in the crypts of jejunum, ileum and colon (see figure 8).

For all measurements samples of ileum, jejunum and mesenteric lymphoid nodes were examined with the light microscope Axioskop 2 plus (Zeiss, Oberkochen, Germany), sections of the colon with the stereomicroscope Stemi 2000-C (Zeiss). Pictures were taken with the AxioCam MRc and measurements were performed with the connected software AxioVision 3.1 (Zeiss).

Total RNA extraction and expression analysis

RNA extraction of whole blood stabilized in paxGene blood RNA tubes was performed using the paxGene blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA extraction of tissue samples was performed with TriFast reagent (Peqlab, Erlangen, Germany). Briefly small pieces of frozen tissue (~50 mg) were cut and immediately immersed in TriFast reagent to avoid RNA degradation. The pieces were then homogenized using MagNA Lyser (Roche Diagnostics GmbH, Penzberg, Germany). The following RNA extraction was performed according to the manufacturer's instructions.

RNA concentration and quality were assessed as described for the *in vitro* investigation.

Primers were chosen using bovine sequences obtained via Ensembl (http://www.ensembl.org/index.html) or NCBI homepage. Primer design and qRT-

PCR runs were performed as described above for the *in vitro* study with the exception that some specific primer sets were adapted from Fleige et al. (2007b and 2009). PCR profiles were modified to meet requirements of each primer pair. Namely the annealing temperatures had to be adapted and if necessary an additional measuring step (15 s at 80°C) was included to avoid problems with primer dimer background signals during quantification. Primer sequences and PCR profile information are given in table 3.

For normalization of mRNA expression results internal standards were chosen for each tissue. This was done by firstly searching for stably expressed genes in different mouse tissues under different conditions. This was done by screening a database containing mRNA micro-array results and using Genevestigator RefGenes (Hruz et al., 2008; Wyss et al., 2010). Mouse tissues were used because at that time no data was available for cattle. Bovine orthologes of stably transcribed genes of intermediate expression levels were chosen for primer design along with commonly used housekeeping genes. C_T values were obtained with Rotor-Gene 6000 software version 1.7 (Corbett Life Science). PCR data of all genes investigated – including all target genes – were used to choose ideal pairs of reference genes for each tissue utilizing GenEx Pro Ver 4.3.4 (multiD Analyses AB, Gothenburg, Sweden). The arithmetic means of C_T values of these pairs were taken for normalization. mRNA expression ratios compared to the control group were calculated for each treatment sample using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical analysis

For each parameter of the histological and haematological analyses the mean group values and the s.e.m. were determined (n=14). For gene expression data fold change results are given in geometric means of expression ratios ± s.e.m. for each group relative to the control group (=1.0).

Statistical analysis of all results was done using the one-way ANOVA method of SigmaStat 3.0. In case of significant differences a post-hoc Tukey-Kramer test was performed to further investigate group differences. Nutritional effects were considered if $P<0.05$. Tendency was considered if $P<0.10$.

Table 3: *Primer pairs, PCR product sizes and appropriate profiles for qRT-PCR in the feeding experiment. For every tissue one pair of reference genes was chosen to normalize crossing point values of target genes*

target		sequence	size	profile
reference genes				
ACTB	for	AACTCCATCATGAAGTGTGACG	234 bp	60/68
	rev	GATCCACATCTGCTGGAAGG		
GAPDH	for	GTCTTCACTACCATGGAGAAGG	197 bp	60/68
	rev	TCATGGATGACCTTGGCCAG		
UBIQ	for	AGATCCAGGATAAGGAAGGCAT	198 bp	60/68
	rev	GCTCCACCTCCAGGGTGAT		
VPS4A	for	CAAAGCCAAGGAGAGCATTC	222 bp	61/68
	rev	ATGTTGGGCTTCTCCATCAC		
GAK	for	TCTGGGAAGTGGCAGAGAGT	294 bp	61/68
	rev	CGGCACGTCTGGTAGAAGAT		
RAB21	for	CGGAAAATGTTGGGAAACG	229 bp	61/68
	rev	CATTGCCTTTTGCCCTCTC		
PMPCA	for	CATCCCAGAATAAGTTTGGACAG	236 bp	61/68
	rev	AGAATCAGCAGACACAGCATACA		
genes of interest				
IL1B	for	TTCTCTCCAGCCAACCTTCATT	198 bp	61/68/80
	rev	ATCTGCAGCTGGATGTTTCCAT		
TNF	for	CCACGTTGTAGCCGACATC	155 bp	61/68/80
	rev	ACCACCAGCTGGTTGTCTTC		
IL8	for	ATGACTTCCAAGCTGGCTGTTG	149 bp	61/68/80
	rev	TTGATAAATTTGGGGTGGAAAG		
TGFB1	for	ACGTCACTGGAGTTGTGCGG	166 bp	61/68/80
	rev	TTCATGCCGTGAATGGTGGCG		
IL10	for	CCTGGAAGAGGTGATGCCAC	118 bp	61/68/80
	rev	GTTTTCGCAGGGCAGAAAGCG		
IFNG	for	CTTGAATGGCAGCTCTGAGAAAC	173 bp	61/68/80
	rev	GGCCTCGAAAGAGATTCTGAC		

*(**Table 3** continued)*

target		sequence	size	profile
reference genes				
FCAR	for	GACAAACCCTTTCTCTCCACC	180 bp	61/68/80
	rev	ACAGGACCCAGAGTGAAGTC		
IL2RA	for	ATGGAGCCAAGCTTGCTGATGT	171 bp	61/68
	rev	TCTGCGGAAGCCTGTCTTGCA		
CD69	for	GTCATTGATTCTAAAGAGGACATGA	137 bp	60/68
	rev	AGGTTGAACCAGTTGTTAAATTCT		
CD4	for	GATCGAGGTCTTGCCTTCAG	237 bp	61/68/80
	rev	GATCTGAGACATCCGTTCTGC		
CD8b	for	ACTGTGTATGGCAAGGAGGTG	127 bp	61/68/80
	rev	GGGTATCCCAATGATCATGCAG		
PECAM1	for	AAGGGAGGCATGACTGTGTC	187 bp	61/68/80
	rev	TAATCACCTCGGACCTGGAG		
EGFR	for	AACTGTGAGGTGGTCCTTGG	173 bp	61/68/80
	rev	AAAGCACATTTCCTCGGATG		
BCL2L1	for	GGCATTCAGCGACCTGAC	203 bp	61/68/80
	rev	CCATCCAAGTTGCGATCC		
BAX	for	TCTGACGGCAACTTCAACTG	203 bp	61/68/80
	rev	GGTGTCCCAAAGTAGGAGAGG		
CASP3	for	GCAACGTTTCTAAAGAAGACCATAG	64 bp	60/68
	rev	CCATGGCTTAGAAGCACACAAATAA		
MKI67	for	TGGCGAAGATGTGTTTCCT	130 bp	60/68
	rev	CGTGCTCCTTGGTGTTTTC		

RESULTS

in vitro investigation: effects of pine pollen extracts on porcine ileal cell culture

Extraction yields

Treatment of shock frozen Masson pine pollen with a ball mill resulted in nearly totally destroyed pollen structures compared to untreated grains (figure 9). The resulting grist was no longer powdery but had a fatty texture from released oily substances. So a good accessibility to pollen compounds for extraction should be guaranteed.

Extraction yields were measured by weighing the vessels for vacuum centrifugation or lyophilization before filling and after completed drying of the extracts. The highest extraction yields were achieved with water and 50% ethanol with 18.3% and 13.7% of total pollen dry matter respectively. Pure ethanol extracted comparably less material with 5.8%. In total 8.4% of pollen dry matter were extracted with the combination of hexane (6.1%) and 80% methanol (2.3%). There seemed to be considerably more water soluble material present in pine pollen than non-polar substances.

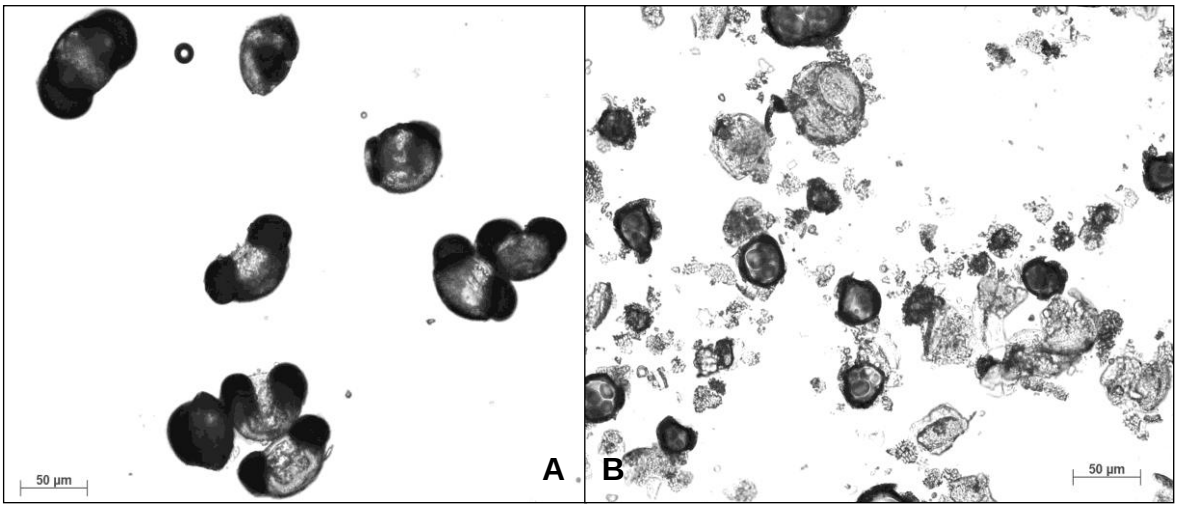

Figure 9: *Masson pine pollen before (A) and after (B) breaking with a ball mill.*

Cell proliferation

After establishment of the ECIS device a high reproducibility of cell proliferation results with comparably little variance within single growth curves in one setup was achieved. The figures 10 to 14 show the mean impedance results of four repetitions for each extract every three minutes. For a better graphic representation only positive s.e.m. for data points every two hours were shown. Standard errors depended on the

magnitude of the impedance values and consequently were increasing with time. Nevertheless they were very homogenously rising so it deems legitimate to show them only for a part of the results. A Student's t-test was performed for each time point – every three minutes – for 48 h. Such a continuous monitoring of cell behaviour could be performed and validated.

A comparison of cells treated with Masson pollen extracts with growth curves of cells treated with the corresponding control extract revealed that water (figure 10) and 50% ethanol extracts (figure 11) decreased normalized impedance values significantly to 70% ($P<0.05$) or 50% ($P<0.05$ to $P<0.01$) of the control values respectively. These differences were statistically significant after 20 h of treatment with the water extract and after 15 h with the 50% ethanol extract. The 100% ethanol extract only transiently influenced development of impedance values (figure 12). Between eight and 24 h after seeding cells treated with the Masson pine pollen extract showed significantly decreased values ($P<0.05$ to $P<0.001$) to 80% of the control treatment results. 80% methanol (figure 13) and hexane pollen extracts (figure 14) did not influence cell proliferation significantly.

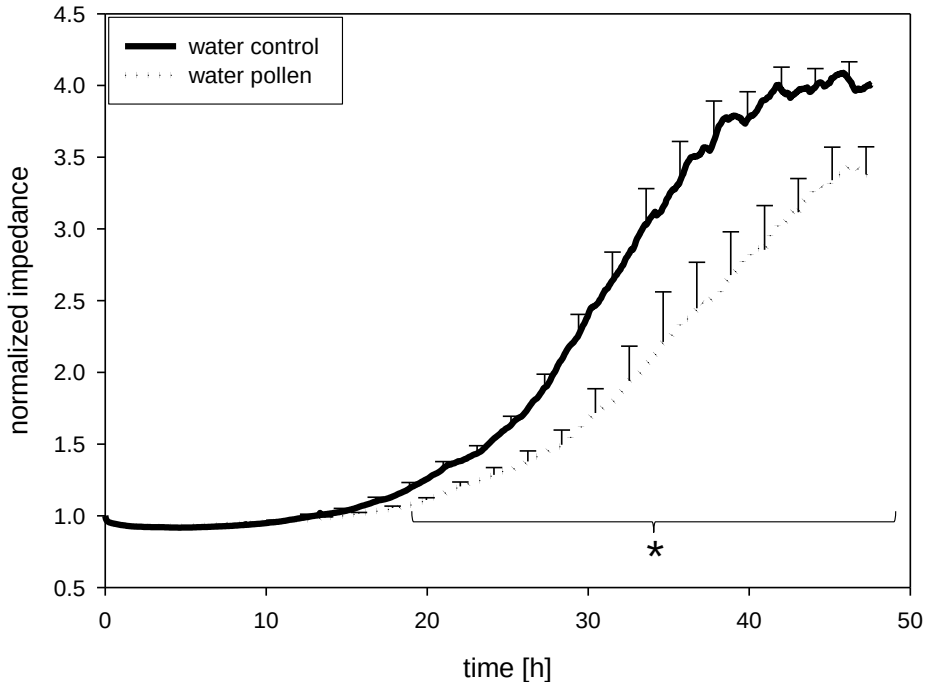

Figure 10: *Water extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium decreased normalized impedance values significantly from approximately 20 h after seeding when compared to cells treated with control extracts. Data is presented as mean + s.e.m. (every two hours). Asterisk shows significant differences (P<0.05).*

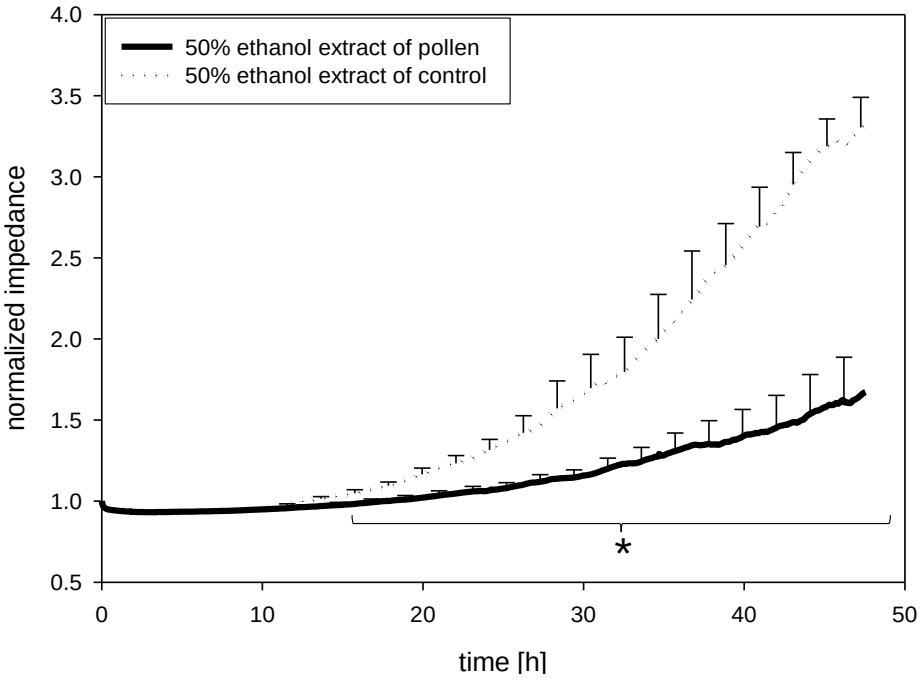

Figure 11: *50% ethanol extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium decreased normalized impedance values significantly from ca. 18 h after seeding when compared to cells treated with control extracts. Data is presented as mean + s.e.m. (every two hours). Asterisk shows significant differences (P<0.05).*

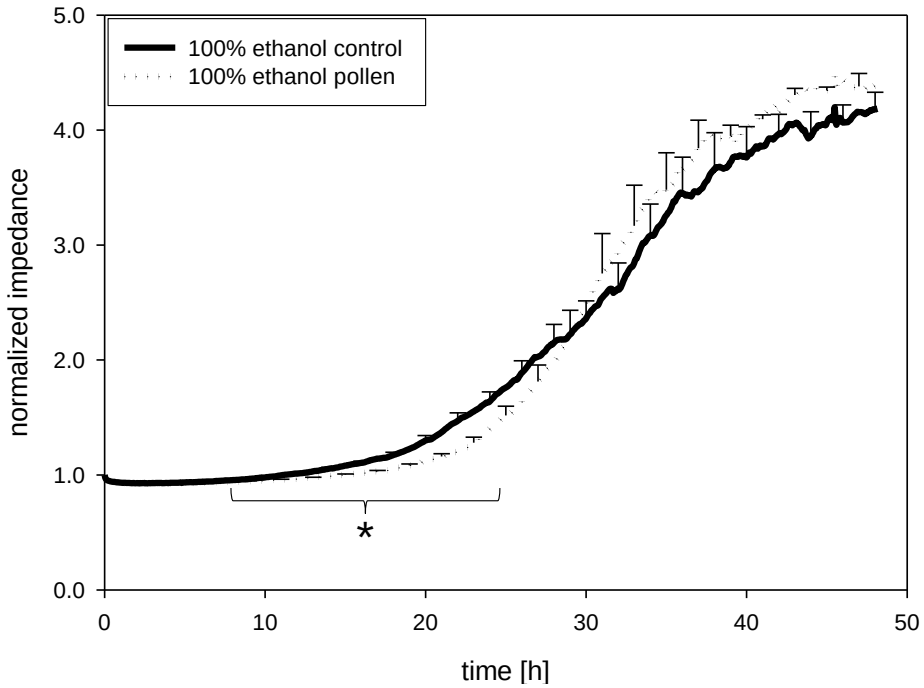

Figure 12: *100% ethanol extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium transiently decreased normalized impedance values significantly from ca. eight to 24 h after seeding when compared to cells treated with control extracts. Data is presented as mean + s.e.m. (every two hours). Asterisk shows significant differences (P<0.05)*

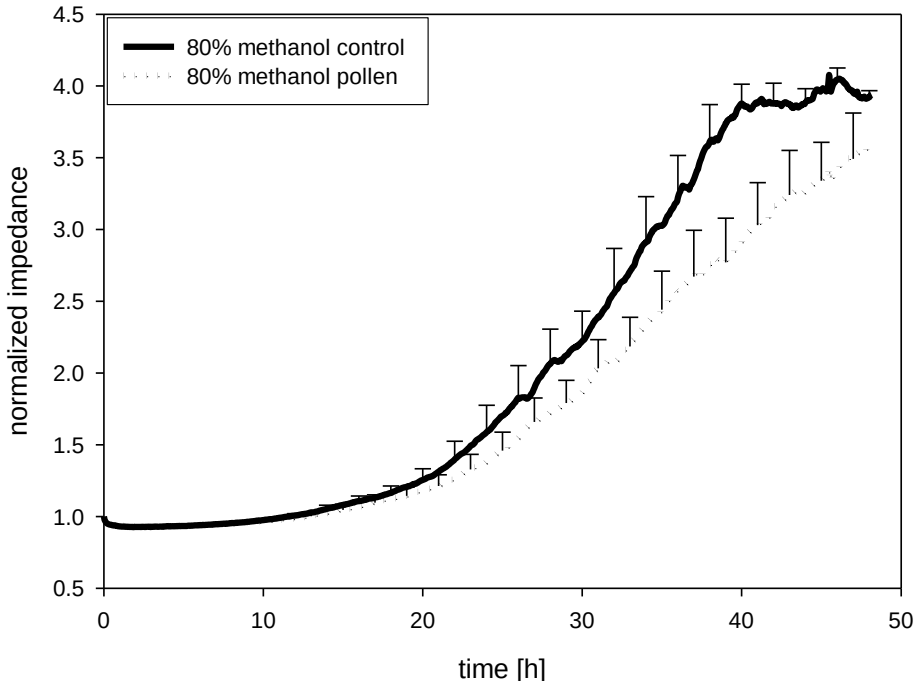

Figure 13: *80% methanol extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium had no significant effects on cell proliferation when compared to cells treated with control extracts. Data is presented as mean + s.e.m. (every two hours).*

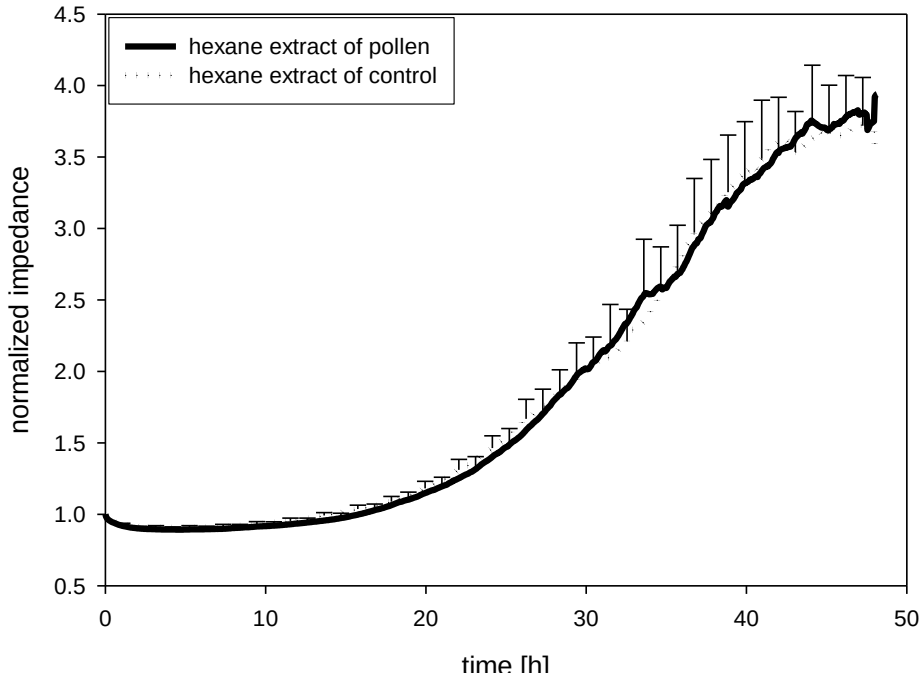

Figure 14: *Hexane extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium had no significant effects on cell proliferation when compared to cells treated with control extracts. Data is presented as mean + s.e.m. (every two hours).*

mRNA expression

RNA purity and quality was found to be excellent as was to be expected with cell culture samples.

Of the five pollen extracts tested only one had statistically confirmed effects on expression of the selected target genes. The 50% ethanol extract of Masson pine pollen led to a significant up-regulation of mRNA expression levels of the pro-inflammatory cytokines IL6 and IL8 to a factor of 4.3 ($P<0.05$) or to factor 2.3 relative to the control level ($P<0.05$) respectively. In the same setup the proliferation regulator CCNA2 was down-regulated to factor 0.7 relative to the control ($P<0.05$). Pro-apoptotic CASP3 and anti-inflammatory TGFB1 were not affected by the treatment with 50% ethanol pollen extract. All these results are summarized in figure 15. The other extracts had no significant effects on mRNA expression levels at a concentration equivalent to 1% whole pollen in the medium (data not shown).

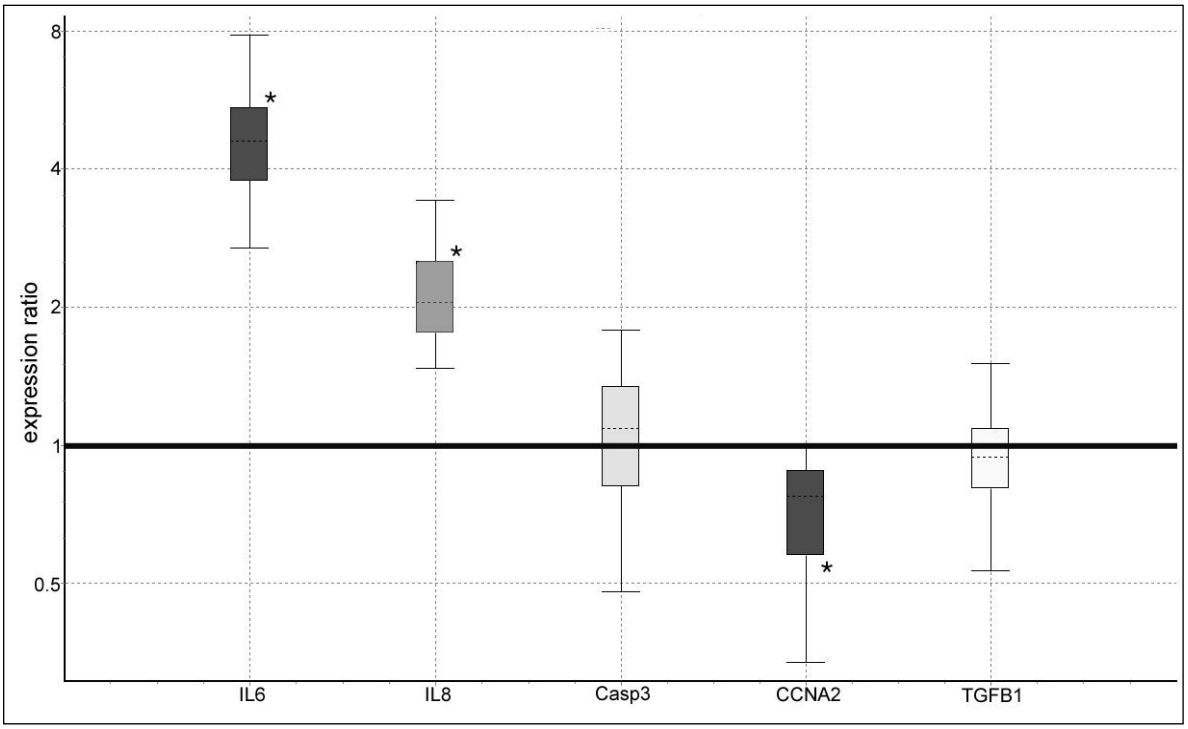

Figure 15: *50% ethanol extract of Masson pine pollen significantly increased relative expression of IL8 and IL6 and decreases the relative expression of CCNA2 compared to cells treated with control extracts without pollen compounds (Asterisks show P<0.05)*

Pollen compounds

A large number of distinct mass signals have been identified with positive and negative charges that are specific for pollen compounds and many of them can be found exclusively in one single extract (table 4). The greatest number of individual

compounds was found in the 50% ethanol extract. At the same time this was the extract with the most signals found exclusively in one extract. In both cases the least signals were found in the hexane extract. Unfortunately none of these substances could be reliably matched to the library of red wine phenolic compounds at the laboratory.

Table 4: *Numbers of LC-ESI-ToF-MS signals specific for Masson pine pollen extracts, that were detected either positively or negatively charged or both. The numbers of substances that are exclusively found in one extract are also given*

extract	**pollen specific substances**			**specific for only one extract**	
	positive	*negative*	*both*	*positive*	*negative*
water	874	308	27	327	101
50% ethanol	1284	671	62	496	266
100% ethanol	740	483	34	218	119
80% methanol	977	513	44	279	125
hexane	373	283	10	166	102

in vivo investigation: effects of inulin and lactulose on preruminant calves

General animal performance

During the feeding experiment general animal performance data was collected and reported previously (Preißinger et al., 2007). Thereby it was found that animals fed inulin had significantly higher daily weight gains (1,269±101 g/d) than animals fed lactulose (1,124±178 g/d, $P<0.05$). Control treated animals ranged between the two experimental feedings with no significant differences to either group (1,203±152 g/d). Interestingly daily ingestion of milk replacer dry matter was very similar between the inulin and control fed calves (1,702±128 g/d and 1,709±117 g/d respectively) while animals of the lactulose group collected significantly less of their daily allowance (1,581±181 g/d, $P<0.05$). The uptake of hay or straw could not be determined for each animal, so total individual feed efficiencies (average dry matter intake/average daily gain) could not be used for statistical analysis. Comparing group averages the inulin group revealed a slightly better ratio (1.55) than control (1.64) or lactulose (1.62).

Haematology

Analysis of blood samples showed that ten weeks after beginning of the feeding experiment animals of the lactulose group had significantly reduced concentrations of peripheral blood leukocytes (P<0.005) compared to inulin and control. The fraction of monocytes of the total leukocytes tended to be decreased by both prebiotics (P=0.07). Both experimental feedings were also able to significantly decrease thrombocyte numbers compared to the control (P<0.01).

When the calves were killed after 20 weeks of feeding all those effects were no longer detectable. Instead both haemoglobin concentration (P<0.005) and haematocrit value (P<0.05) were significantly increased in the inulin group compared to control and lactulose. A similar pattern could already be seen at ten weeks but was then not statistically confirmed (P=0.29 or P=0.16 respectively).

All results of blood investigations are listed in table 5.

Table 5: *Results of haematological examination of blood samples taken ten weeks after beginning or during slaughtering. All values are presented as mean±s.e.m. (n=14). P values are results of ANOVA analysis. Within rows values without a common superscript additionally have been found significantly different by post hoc Tukey-Kramer test (P<0.05)*

after ten weeks		control	inulin	lactulose	P value
erythrocytes 10^6 µL^{-1}		8.8 ± 0.34	9.2 ± 0.22	8.9 ± 0.28	n.s.
thrombocytes 10^3 µL^{-1}		0.75 ± 0.046^a	0.55 ± 0.033^b	0.66 ± 0.062^b	0.008
leukocytes 10^3 µL^{-1}		8.9 ± 0.53^a	9.6 ± 0.84^a	6.5 ± 0.51^b	0.005
granulocytes [%][1]	basophil	0.9 ± 0.10	0.6 ± 0.14	0.6 ± 0.13	n.s.
	eosinophil	2.4 ± 0.52	2.1 ± 0.45	2.4 ± 0.53	n.s.
	segmented	28.2 ± 2.18	31.3 ± 2.57	31.6 ± 2.55	n.s.
lymphocytes [%][1]		61.4 ± 2.27	61.4 ± 3.25	60.1 ± 3.01	n.s.
monocytes [%][1]		6.4 ± 0.66	4.1 ± 0.75	4.8 ± 0.76	0.073
hemoglobin [g/dL]		7.1 ± 0.38	7.5 ± 0.29	6.8 ± 0.33	n.s.
hematocrit [%]		17.1 ± 0.82	18.6 ± 0.85	16.4 ± 0.76	n.s.

*(**Table 5:** Continued)*

after 20 weeks		control	inulin	lactulose	P value
erythrocytes 10^6 µL^{-1}		11.2 ± 0.41	12.2 ± 0.31	11.8 ± 0.29	n.s.
thrombocytes 10^3 µL^{-1}		0.46 ± 0.103	0.58 ± 0.074	0.65 ± 0.074	n.s.
leukocytes 10^3 µL^{-1}		5.3 ± 0.51	6.2 ± 0.56	6.9 ± 0.85	n.s.
granulocytes [%][1]	basophil	0.6 ± 0.15	0.6 ± 0.14	0.8 ± 0.17	n.s.
	eosinophil	3.5 ± 0.81	3.8 ± 0.56	3.5 ± 0.85	n.s.
	segmented	41.2 ± 4.40	42.9 ± 3.89	47.6 ± 2.91	n.s.
lymphocytes [%][1]		49.7 ± 4.90	47.9 ± 3.64	42.6 ± 3.51	n.s.
monocytes [%][1]		4.6 ± 0.74	4.5 ± 0.53	4.5 ± 0.60	n.s.
hemoglobin [g/dL]		7.7 ± 0.36[a]	8.7 ± 0.27[b]	8.1 ± 0.54[a]	0.003
hematocrit [%]		21.8 ± 0.85[a]	24.2 ± 0.72[b]	21.6 ± 0.76[a]	0.035

[1]of total leukocytes

Histological analyses

In jejunum villus length tended to be decreased in inulin and increased in lactulose treated animals when compared to the control group (P=0.07). Statistically significant changes of villus lengths in the same directions could be found in ileum (P<0.05). There the post-hoc Tukey-Kramer test revealed statistically confirmed differences between the two experimental feeding groups (P<0.05) but not between either of them and the control group. Neither villus width in jejunum and ileum nor crypt depth or distance between crypts in colon was significantly affected by the diet.

Similar regulations were found for the densities of cells positive for the proliferation marker MKI67 in ileum. There a tendency was found for inulin to decrease and for lactulose to increase the number of proliferative cells (P=0.08). In jejunum and colon no significant changes could be confirmed.

In jejunum and ileum densities of goblet cells were investigated individually for crypts and villus tips. In ileal villus tips but not in crypts their numbers tended to decrease (P=0.07) in both experimental treatments. In jejunum no effects could be found.

No diet related changes could be found for area and densities of lymph follicles in mesenteric lymphoid nodes and ileal Peyer's patches.

All results of histological analyses are summarized in table 6.

Table 6 *Results of measurements on mucosal architecture, goblet cell numbers, lymphoid follicles in Peyer's patches and mesenteric lymphoid nodes (n=14) and on cell proliferation (n=6) (all values are demonstrated as mean±s.e.m.). P values are results of one way ANOVA. Within rows values without a common superscript have been found significantly different after post hoc Tukey-Kramer test (P<0.05)*

	treatment groups			**P value**
	control	*inulin*	*lactulose*	
villus height [mm]				
jejunum	0.77±0.13	0.71±0.11	0.84±0.18	P=0.07
ileum	0.69±0.16^{ab}	0.59±0.15^{a}	0.78±0.21^{b}	P=0.03
villus width [mm]				
jejunum	0.13±0.01	0.13±0.03	0.12±0.02	n.s.
ileum	0.11±0.02	0.10±0.02	0.12±0.03	n.s.
crypt depth [mm]				
colon	0.42±0.06	0.40±0.09	0.42±0.08	n.s.
distance between crypts [mm]				
colon	0.02±0.01	0.02±0.01	0.03±0.01	n.s.
goblet cells (villus tip) [mm^{-1}]				
jejunum	6.2±4.3	10.2±6.0	8.0±6.6	n.s.
ileum	25.9±11.4	17.3±8.5	15.8±11.8	P=0.07
goblet cells (crypt) [mm^{-1}]				
jejunum	28.0±11.7	29.5±9.5	30.3±13.7	n.s.
ileum	70.6±14.3	61.5±10.2	61.3±14.2	n.s.
proliferative cells [mm^{-1}]				
jejunum	59.7±25.8	51.0±25.3	62.0±26.0	n.s.
ileum	45.9±21.6	32.0±12.3	59.4±22.4	P=0.08
colon	26.4±16.8	13.1±5.7	19.0±10.4	n.s.
lymphoid nodes				
follicle area [mm^2]	0.07±0.03	0.07±0.01	0.07±0.02	n.s.
follicle density [mm^{-2}]	1.80±0.54	1.88±0.68	1.77±0.53	n.s.
Peyer's patches				
follicle area [mm^2]	0.08±0.03	0.09±0.05	0.07±0.03	n.s.
follicle density [mm^{-2}]	7.04±1.76	6.89±2.71	7.03±2.54	n.s.

RNA quality

RNA extraction of tissue samples sometimes is challenging and it is not always possible to get highly pure and integer material. In the present study RNA purity was regarded as good with an average A260/A280 absorption ratio of 2.0±0.01 for all tissues. This is considerably better than 1.8 which is suggested to be an indicator for high purity. RNA quality yielded an average RNA integrity number (RIN) of 6.9±0.13 across all tissues. RNA quality described by RIN ranges from totally degraded (RIN=1.0) to absolutely intact RNA (RIN=10.0). Single samples that had no sufficient quality for qRT-PCR were re-extracted. If values did not reach adequate levels after re-extraction from different backups the samples were omitted from analysis.

Choice of reference gene pairs

Reference gene candidates are acceptable if their mRNA expression in one tissue is stable throughout all treatment groups that should be compared with each other. Screening of all PCR data revealed that there were no reference genes that were ideal for all tissues used. So for each tissue an individual pair of normalizers was chosen using GenEx Pro software (MultiD Analyses AB). Those are given in the following:

jejunum:	ACTB and GAK
ileum:	VPS4A and GAK
colon:	UBIQ and GAPDH
spleen:	RAB21 and PMPCA
blood:	ACTB and UBIQ

mRNA expression changes of inflammation and immune modulating factors

In jejunum lactulose led to a significant down-regulation of the pro-inflammatory TNF ($P<0.05$). Post-hoc Tukey-Kramer test showed that this effect was statistically confirmed only between inulin and lactulose ($P<0.05$), but not between inulin and control.

Expression of IL10 in ileum was also influenced by the diet ($P<0.05$). Its relative mRNA amount was increased by inulin and decreased by lactulose, both compared to the control. Thereby the difference between the two experimental feeding groups was statistically confirmed ($P<0.05$), while the discrepancy between each of them and the control was not.

In the large intestine the relative expression of PECAM1 was enhanced by both prebiotic feedings (P<0.05). No difference could be detected between inulin and lactulose, but between each of the two groups and the control (both P<0.05). In the same intestinal segment the mRNA amount of IL2RA tended to be decreased by lactulose (P=0.058).

In mesenteric lymphoid nodes the chemo-attractant IL8 was significantly up-regulated by lactulose (P<0.05) compared to control and to inulin (both Tukey-Kramer test P<0.05). All significant regulations are summarized in table 7.

Expression of IL1B, TGFB1, CD69, CD4 and CD8B were not influenced by the feeding group in any tissue analyzed. Investigation of spleen and blood did not reveal any regulation of gene expression (data not shown).

mRNA expression changes of proliferation and apoptosis related genes

Changes in the expression of proliferation or apoptosis related genes were only found in ileum. There the proliferation marker MKI67 was influenced by the feeding group (P<0.01). Its expression was significantly up-regulated by inulin compared to lactulose (P<0.01) and tended to be increased compared to the control (P=0.069). Both prebiotic feedings showed a weak tendency to up-regulate expression of pro-apoptotic BAX (P=0.096) in the same tissue. BLC2L1, EGFR and CASP3 were not influenced by the feeding regime. These results are also listed in table 7.

Table 7: *Changes in relative mRNA expression levels found in the* in vivo *study. All values are presented as geometric mean±s.e.m. (n=14) of relative expression values with respect to the control levels. P values are results of ANOVA analysis. Within a row means without a common superscript have been found significantly different after post-hoc Tukey-Kramer test (P<0.05)*

tissue	gene	control	inulin	lactulose	P value
jejunum	TNF	1.0 ± 0.11^{ab}	1.1 ± 0.11^{b}	0.7 ± 0.10^{a}	0.018
ileum	IL10	1.0 ± 0.19^{ab}	1.5 ± 0.17^{b}	0.8 ± 0.20^{a}	0.048
ileum	BAX	1.0±0.10	1.3±0.10	1.3±0.10	0.096
ileum	MKI67	1.0 ± 0.27^{ab}	2.0 ± 0.35^{b}	0.8 ± 0.15^{a}	0.009
colon	PECAM1	1.0 ± 0.06^{a}	1.3 ± 0.11^{b}	1.3 ± 0.14^{b}	0.023
colon	IL2RA	1.0±0.20	0.9±0.32	0.7±0.15	0.058
lymph node	IL8	1.0 ± 0.17^{a}	1.0 ± 0.10^{a}	1.4 ± 0.15^{b}	0.029

DISCUSSION

in vitro investigation: effects of pine pollen extracts on porcine ileal cell culture

Masson pine pollen is one of the products newly emerging in the western world in the course of traditional Chinese medicine. Its health promoting effects are heavily advertised and certain effects on gut health of weaning piglets have already been found (Schedle et al., 2008), that have been proposed to be of prebiotic character. To investigate if further effects independent of prebiotic activities on gut flora may also play a role a combination of real-time cell growth monitoring, qRT-PCR and LC-ESI-ToF-MS was used to find possible effects of different soluble pollen compounds on a porcine ileal cell culture.

Extraction yields

Extraction yields from Masson pine pollen with the method used in the present study (up to 18.3% w/w for the water extract) were relatively similar to the yield obtained by Choi (2007). There extraction was done with intact *P. densiflora* pollen and with 70% ethanol for three days at room temperature and resulted in a yield of 8%. But since the *P. massioniana* pollen in the present work was broken prior to extraction it may be expected that all pollen compounds are extracted equally regardless of their position within the pollen. With intact pollen this grade of accessibility may not have been granted.

Cell proliferation

Establishment of the ECIS system enabled monitoring of the behaviour of a cell culture with very short intervals between measurements and a very high reproducibility. Concretely four repetitions each of one extract and its control respectively could be run on one array with measurements every 3 min until RNA extraction after 48 h. This is a resolution that could not be achieved with conventional cell culture techniques. For each time point a t-test was performed which gives a total of some 960 single statistical tests.

Most of the work on pollen effects is done in feeding experiments. Only little data is available on effects of pollen extracts on cell cultures. In the present study pine pollen extracts soluble in 80% methanol or hexane had no effects on cell

proliferation. But material soluble in water or ethanol or a mixture of them did show transient or overall lowering effects on cell proliferation. In contrast to these results a piglet feeding study revealed that addition of whole *P. massoniana* pollen led to increased villus heights in the small intestine (Schedle et al., 2008) suggesting an enhanced cell proliferation. But since whole pollen was fed it cannot be determined whether these effects are caused by molecules present in our extracts or insoluble material. Furthermore effects of pine pollen in feeding experiments are rather attributed to their character as an insoluble fibre due to cell wall components (Zhao et al., 1996) which may have overruled opposing actions of other pollen substances in the piglets. The extraction procedure should have eliminated non-soluble components nearly totally and since no intestinal flora was present in the cell culture setups no prebiotic effect of other components could have been present.

In other studies bioactive properties of bee pollen (Kroyer and Hegedus, 2001) as well as from extracts of *P. densiflora* pollen (Choi, 2007) have been attributed to their high content of polyphenolic substances that has also been reported for pine pollen (Strohl and Seikel, 1965). Different pure polyphenols or mixtures of them from a variety of plants have been reported to decrease cell proliferation in several cell lines (e.g. Kaneuchi et al., 2003; Briviba et al., 2002, Ijima et al., 2000). So these compounds of Masson pine pollen could be an explanation of the decrease in cell proliferation observed in the present study. Because different types of polyphenols show characteristic behaviour towards a variety of solvents it can be possible that not all extracts used in the present study did show similar effects.

mRNA expression

Two of the extracts – water and 50% ethanol – had very similar decreasing effects on cell proliferation. But interestingly of all extracts only the 50% ethanol extract did show significant influences on mRNA expression of the target genes. The down-regulation of the proliferation regulator CCNA2 could be an explanation for the decrease in cell proliferation observed. But it is to be expected that additional proliferation or apoptosis regulators are engaged in this process since no effect on CCNA2 expression was visible in the water extract. The expression of CCNA2 itself may be influenced by phenolic compounds that are soluble in 50% ethanol but less in water or one of the other solvents used for extraction. A down-regulation of its expression by red wine polyphenols has been proven before (Iijima et al., 2000).

Up-regulation of IL6 and IL8 suggests that pine pollen substances are able to change the inflammatory status of ileal cells. *In vivo* IL6 would play a role in gut barrier formation, immune regulation by effects on T and B cell differentiation, acute phase reaction including stimulation of other pro-inflammatory cytokines and in local inflammation (Castell et al., 1988; Dienz and Rincon, 2009; Romano et al., 1997; Song and Kellum, 2005). On the other hand in recent years IL6 has been discussed to have anti-inflammatory properties by controlling simultaneously induced pro-inflammatory mediators possibly either in their production or in their receptor interaction (Tilg et al., 1994; Xing et al., 1998). However the results of the present study contradict a study performed by Leiro et al. (2005) who found a decrease of IL6 expression in LPS activated macrophages treated with extracts from *P. densiflora*.

IL8 is a chemotactic protein that attracts neutrophils to sites of inflammation and activates them – e.g. in ulcerative colitis and Crohn's disease (Mitsuyama et al., 1994). So it is clearly considered as a pro-inflammatory molecule. Interestingly expression of IL8 can be increased by reactive oxygen species such as hydrogen peroxide or the hydroxyl radical and can be decreased by anti-oxidative substances (DeForge et al., 1993) such as polyphenols. Also the anti-inflammatory and radical scavenging effects of *P. densiflora* pollen found by Choi (2007) and Lee et al. (2009) have been attributed to their content of flavonoids which are commonly reported to have anti-oxidative activities (Rice-Evans et al., 1996). Substances of this group have even been found to block hydrogen peroxide induced IL8 secretion (Matsuoka et al., 2002). Additionally IL8 is a pro-inflammatory signal while polyphenols mostly have been found to have anti-inflammatory properties (e.g. Choi, 2007, Porath et al., 2005). All in all these results would suggest that the effects observed are not caused by the polyphenolic contents of *P. massoniana* pollen. But nevertheless evidence exists for some of these substances – e.g. *cis*-resveratrol – to increase expression of inflammation mediators such as IL6 and TNF (Leiro et al., 2005).

Pollen compounds

Analysis of *P. massoniana* pollen extracts found a multitude of mass signals specific for pollen extracts in general or even specific for one single extract. Some of these mass signals can only be found in extracts that did reveal effects during the cell culture experiments and not in 80% methanol or hexane extracts. These mass signals are candidates for causing the effects found in the present study.

Unfortunately these substances could not be matched to a library of phenolic compounds present in red wines that was established in the laboratory of Dr. Thomas Letzel. This may be due to differences in the matrix between pollen extracts and red wines that possibly influence behaviour of the molecules during chromatography and ionization. However, most compounds could be characterized via retention factor (hydrophobicity), ionization characteristics or the molecular formula by comparing it to a forthcoming database (Berkemeyer and Letzel, 2008). If at least a subset of these molecules could be identified, effective extract specific substances could be tested at appropriate concentrations in cell culture to investigate whether they are able to cause effects found with pollen extracts. But it has to be expected that these effects are not caused by single substances, but by a combination of agents. This would explain similar but not totally identical effects of water and 50% ethanol extracts. Anyway a first concentration on mass signals that are present in these two effective extracts and absent in at least the two totally non-effective ones would narrow the spectrum of possible bioactive compounds to a number of ca. 150. Although effects of pollen are often discussed to be mediated by their flavonoid content the causing agents may not all be of polyphenolic character. Pine pollen have also been found to contain a variety of lipids (Scott and Strohl, 1962) or polysaccharides (Bouveng, 1963) that may also have effects on cell proliferation or gene expression (e.g. Yang et al., 2007).

in vivo investigation: effects of inulin and lactulose on preruminant calves

A lot of literature is available on prebiotics, often in combination with probiotic nutrition. But most of it is concentrating on human nutrition or laboratory animals that serve as model for human hosts. Comparably little has been published on behalf of prebiotic feeding in farm animals although prebiotics are considered as one possible alternative to antimicrobial growth promoters to improve animal health and performance. Even less literature is available on prebiotic feeding experiments with calves and most of these studies concentrate on animal performance or intestinal bacteria populations (Flickinger et al., 2003; Heinrichs et al., 2009) without much attention on intestinal morphology, immunology or gene expression changes. The present study is meant to close some gaps on the action of two commonly used

prebiotics on pre-ruminant calf health with respect to intestinal mucosa and gut associated immune system.

General animal performance

Influences of inulin and oligofructose on animal performance have been investigated in a variety of studies. Quite a number of them found no or only little effects on performance characteristics, but some reported effects similar to those found in the present study. Feeding inulin or oligofructose there led to improved daily weight gains and feed efficiencies (Flickinger et al., 2003; Kaufhold et al., 2000; van Loo, 2007). Lactulose has also been considered as a prebiotic. But only little data on animal performance has been available up until now. Fleige et al. (2007) found a significant increase in feed consumption and a tendency for an improvement of daily weight gains of calves after feeding 3% lactulose with their milk replacer. But since a probiotic strain was added to the milk replacer a modulation of lactulose actions could be possible.

The differences between the lactulose and the control group may be based on differences in energy uptake due to a lower ingestion of milk replacer. The feed efficiency is very similar between these groups showing no additional influence of lactulose feeding beside a lessened appetite. But although inulin and control fed animals have a very similar milk replacer consumption the feed efficiency is better in the prebiotic group. This clearly shows a boosting effect of inulin on animal performance.

Haematology

Up until now only one other long-term study with lactulose feeding to calves was performed where haematology was investigated. There the authors found an increase of lymphocyte numbers in animals fed 1% lactulose and a decrease of thrombocyte numbers with 3% lactulose (Fleige et al., 2009). The latter finding could be supported by the findings for both prebiotics in the present study. Here thrombocyte concentrations were significantly decreased after ten weeks of feeding. Such a lowering may also decrease thrombocyte aggregation which is considered one of many risk factors for coronary heart disease. A lowering of platelet aggregation by intakes of higher amounts of dietary fibre has been found before in rats (Bagger et al., 1996). This effect may be one of several possible explanations for

the results of a study that found an inverse correlation between intake of fibre rich food and the incidence of coronary heart disease (Pietinen et al., 1996).

At slaughtering only haemoglobin concentration and haematocrit were affected by the diet. Inulin led to an increase of both parameters which may be caused by an improvement of iron absorption. Similarly Ohta et al. (1998) reported that both values were decreased after gastrectomy with subsequent iron malabsorption in rats, but could be recovered by feeding of 0.75% oligofructose. Veal calves that are fed solely milk replacer without supplementation of dietary fibre or additional solid feed often have been found to be iron deficient which can lead to reduced daily gain and more infections (Cozzi et al., 2002; Le Neindre, 1993). Also in the present study all group means for haematocrit and haemoglobin concentration were below the normal range given by the investigating laboratory (28-38% or 9-14 g/dL respectively) and all calves had to be treated with iron supplementation during the feeding period. It is possible that inulin – but not lactulose – feeding was able to improve iron supply since it was found to increase the fraction of soluble iron in the caecum of rats and so improved haemoglobin concentration and haematocrit values similar to the present study (Ohta et al., 1995). Nevertheless values were still not in an optimal range for calves but significantly better than in the other feeding groups.

Modulatory effects of prebiotic substances on the immune system and inflammatory reactions have been found before, especially for inulin and oligofructose (Schley and Field, 2002; Watzl et al., 2005). Amongst others influences were found on the composition and function of the gut associated lymphoid tissue for example in dogs that were fed a fibre rich diet (Field et al., 1999). In the present study haematological traits were investigated and a decrease of leukocyte counts was found in lactulose treated calves. Similar results have been found with mannanoligosaccharides from yeast cell wall preparations fed to dogs (Middelbos et al., 2007). In this study the prebiotic treatment also decreased monocyte numbers similar to the results found for both experimental feeding groups in the present study. The authors suggest that mannan moieties can lead to a lowering of the infectious load in the dogs' intestines which may in turn decrease the necessity of a high number of active immune cells in the gut. This again may be reflected in peripheral blood. This is consistent with the finding that synbiotic enteral nutrition after hepatectomy also decreased intestinal pathogen load of patients and improved post-surgery infectious complications including white blood cell counts compared to standard treatment (Kanazawa et al.,

2005). Prebiotics such as inulin or lactulose are attributed to lead to a comparable decrease in pathogen load in the intestine (Bovee-Oudenhoven et al., 1997; Flickinger et al., 2003). Nevertheless the animals were healthy in the present study and no immune reaction was evident in the other groups so a lowering of leukocyte counts may not necessarily be a positive sign of lowered infectious load. It also could be a hint to a mild leukocytopenia or, in combination with the lowered thrombocyte counts, to symptoms of a sub-clinical aplastic anemia. But up until now there are no reports on unwanted side effects of lactulose on haematological traits.

Both prebiotics had the same or very similar effects on thrombocyte concentration and monocyte proportion but differed clearly in their impact on leukocyte counts and haemoglobin and haematocrit suggesting different modes of action of the two substances possibly associated with their specific effects on bacterial subpopulations (Rycroft et al., 2001). Nevertheless, a decrease in leukocyte counts not necessarily has to be interpreted as a positive effect, but an increase of iron absorption and possibly also uptake of other trace elements by inulin consumption is clearly able to improve animal health.

Histological analyses

As discussed above animals fed inulin had a significantly higher daily weight gain and a better feed conversion ratio. Interestingly these facts are commonly associated with a better intestinal nutrient absorption due to longer villi (Awad et al., 2008; Wu et al., 1996). But quite in contrary in the present study small intestinal villi were shorter in animals fed inulin than in the other groups. A similar shortening of villi caused by inulin feeding has been reported in weaning piglets (Pierce et al., 2005). On the other hand increases of villus length after feeding soluble chicory extract or purified inulin have been reported, too (Kim, 2002). Supporting evidence for the shortening of villi exists in the decrease of MIB1-positive and such considered proliferative cells in ileum of inulin fed calves. An anti-proliferative effect of relatively high concentrations of inulin and oligofructose (10% of the diet) has been reported before in rats and was considered anti-tumorigenic (Femia et al., 2002). In summary it may be considered that a decelerated proliferation rate resulting in shorter villi may reduce the energy demand for maintenance of gut architecture. This would in turn provide more energy for growth and fattening and may so contribute to an improved animal performance. A further lowering of energy requirements may also have been achieved by a

decrease of goblet cell numbers in ileal villus tips also found in inulin fed animals via a decreased mucus secretion effort. The latter finding stands in contradiction to an increase of goblet cell densities in rats fed inulin or other non-starch polysaccharides (Kim, 2002). On the other hand it is supported by a decrease of their number in piglets fed a diet with the carob tree seed meal as source for prebiotic galactomannans (Van Nevel et al., 2005).

When contemplating the results for villus length and proliferation in the lactulose group one can see that there opposing effects occur. Although one would expect the opposite considering the decrease in daily weight gains villus heights are increased in this group. This stands in contrast to the results of Fleige et al. (2007a), who found a shortening of villi in calves fed lactulose in combination to a probiotic bacteria strain. But again the action of the synbiotic treatment may differ from a solely prebiotic effect. In accordance to the longer villi found here lactulose also tended to promote proliferation in ileum which is similar to actions of other dietary fibres such as guar gum or pectin. These substances were found to increase the proliferation zone in the caecal crypts of rats (Brunsgaard and Eggum, 1995). It is noteworthy that although the nutrient ingestion was decreased to ~90% of the control value still the villus length and proliferation rate increased. This may not be expected when considering studies on fasted animals (Clarke, 1975). But in this case animals were starved for short times while calves only experienced a slight energy restriction and another study found no reduction in villus height in pigs with a much stronger energy restriction (50%) compared to a normally fed control (Claus et al., 2006). Commonly it is expected that an improved intestinal morphology with longer villi leads to an improvement in animal performance (Awad et al., 2008; Wu et al., 1996) but this may be overruled by the lowered energy ingestion in the lactulose group. Nevertheless similar to inulin lactulose led to a decrease in goblet cell density in villus tips in ileum.

The differences in effects on mucosal architecture between the treatment groups are not easily explained by differences in nutrient consumption alone. A possible explanation for these differences in gut mucosal architecture could be different sites of action due to divergent fermentation properties. And indeed it has been found in pigs that pre-caecal fermentability of lactulose was lower than that of inulin (Branner et al., 2004). Additionally specific stimulatory effects on beneficial bacterial subpopulations and fermentation product profiles were reported (Rycroft et al., 2001). Similar effects such as the decrease of ileal villus tip goblet cell numbers might be

regulated by other factors that may be similar between both prebiotics, such as viscosity that is able to influence goblet cells (Ito et al., 2009).

mRNA expression changes of inflammation and immune modulating factors

Changes of the expression of immunological and inflammatory mediators or markers after feeding prebiotic substances have been reported frequently. For example Hosono et al. (2002) found an increase of IgA and an altered cytokine expression pattern in murine Peyer's patch cells after feeding FOS to the animals. During hematologic investigation a decrease of leukocyte numbers was found in the blood of lactulose treated calves. Supporting this finding the expression of IL2RA – an activation marker on the surface of lymphocytes (Lai et al., 1998) – tended to be lowered in the colon of lactulose treated calves compared to the control. This result is also consistent with a report by Fleige et al. (2009) who found a significantly lowered expression of IL2RA (CD25) in mesenteric lymph nodes of lactulose treated calves. Not only leukocyte counts and IL2RA expression in colon were significantly reduced by lactulose, but also expression of TNF in jejunum. This supports a finding made in a rat colitis model after treatment with lactulose (Camuesco et al., 2005). All these findings could be explained by a reduction of the pathogenic load in the intestine leading to lowered lymphocyte activation and a decreased activation of inflammatory responses. But in contrast to the latter finding the pro-inflammatory chemokine IL8 was significantly increased in mesenteric lymphoid nodes of animals in the lactulose group contradicting a decrease of inflammatory responses in the vicinity of the intestine. Fleige et al. (2009) reported similar observations with an increase of IL8 mRNA amounts in mesenteric lymphoid nodes of lactulose fed calves, but their results were not statistically confirmed. Expression of IL8 has been shown to be influenced by lipoteichoic acid of gram positive bacteria (Standiford et al., 1993). Bacteria that are promoted by lactulose include gram positive bifidobacteria (Camuesco et al., 2005). So IL8 expression could be influenced by antigens of these bacteria transported to the lymphoid nodes by antigen presenting cells. But in fact no effect on IL8 could be detected in gut tissue where such a signal should become evident in the first since bifidobacteria and lactobacilli have been shown to be able to induce IL8 expression in an epithelial cell line (Lammers et al., 2002). Additionally inulin also increases the number of bifidobacteria in the gut (Campbell et al., 1997), but did not lead to similar effects on IL8 expression. All in all it is questionable

whether other factors possibly independent of lactulose effects on the gut flora may be responsible for the IL8 response observed.

Expression of anti-inflammatory IL10 was increased in the ileum of inulin treated calves. The ileal wall is interstratified with Peyer's patches, which are part of the gut associated lymphoid tissue. These patches or cells thereof have been shown to release anti-inflammatory cytokines including IL10 when stimulated with probiotics, prebiotics or their fermentation products (Hosono et al., 2003; Roller et al., 2004; Säemann et al., 2000). Interestingly only inulin had this anti-inflammatory effect on ileal tissue while lactulose had a rather lowering effect on IL10 expression. This is consistent with results of a study by Fleige et al. (2009) where a slight down-regulation of ileal IL10 was found in lactulose treated calves. Contrastingly during the same experiment the authors also found an increased expression of IL10 in the jejunum and colon of said animals (Fleige et al., 2007b). Since IL10 is able to inhibit cytokine secretion, e.g. of TNF, and effects of macrophages, natural killer cells and T cells (Moore et al., 1993) an effect on local and possibly systemic inflammatory and immune response can be expected but interestingly no effects on TNF or IL8 could be detected in the same intestinal segment in inulin fed animals.

Another interesting molecule influenced by prebiotic feeding is PECAM-1, a cell surface molecule of leukocytes and endothelial cells. This molecule is crucial for the transmigration of white blood cells through capillary walls to reach sites of inflammation (Wakelin et al., 1996). Regarding this fact alone an up-regulation of its expression, as was found in the colon of animals of both prebiotic fed groups, would be a pro-inflammatory event. But surface expression of PECAM-1 as well as junctional adhesion molecule (JAM) on endothelial cells has also been shown to be down-regulated after stimulation with TNF or IFNG, typical pro-inflammatory signals. Contradicting the study of Wakelin et al. (1996) leukocytes still were able to pass freely between the cells although less PECAM-1 was present and only monocyte transmigration was reduced. The decrease of PECAM-1 and JAM increased permeability of the endothelial wall (Shaw et al., 2001). This suggests that an increased expression may strengthen the barrier function of endothelial cells and decrease migration of immune cells. Additionally PECAM-1 also seems to be involved in the regulation of angiogenesis (DeLisser et al., 1997). So an increased expression may also result in increased perfusion of the surrounding tissue. Maybe an up-regulation of PECAM-1 could be an explanation for the finding that a probiotic

strain, *L. rhamnosus*, was able to enhance gastric ulcer healing by stimulation of angiogenesis (Lam et al., 2007). Another commensal bacteria strain, *B. thetaiotaomicron* was also found to enhance expression of angiogenin-3, an angiogenic factor (Hooper et al., 2001). So beneficial bacteria promoted by the feeding of inulin or lactulose could also influence perfusion of gut tissue. The only study that investigated effects of a prebiotic substance – lactulose – on PECAM-1 expression until now did not lead to any significant results (Fleige et al., 2009). So this is a novel finding of prebiotic actions and should be interpreted with care until further information is available.

Both prebiotics are able to influence expression of certain molecules involved in inflammation or immune regulation or even possibly wound healing. But again the effects of both substances are rather diverse. While lactulose rather decreases pro-inflammatory signals, with the exception of IL8, inulin increases anti-inflammatory signalling. Also the sites of action within the intestinal tract differ. Lactulose acts on jejunum and ileum while inulin acts on ileum and colon, hinting to different fermentation kinetics.

mRNA expression changes of proliferation and apoptosis related genes

Inulin led to an increased ileal expression of the proliferation marker MKI67. Interestingly this contradicts the results obtained during morphological investigation where a slight decrease of villus length and number of proliferative cells was found in the same group. The increase of both parameters in the lactulose group is also not reflected in expression of MKI67. This may be caused by a concentration on the ileal mucosa during morphological investigation while qRT-PCR was performed with whole intestine tissue samples. Regulation in deeper layers of the gut tissue may be different from top layers. Nevertheless, both stimulation (Brunsgaard and Eggum, 1995) as well as decrease (Femia et al., 2002) of gut epithelial proliferation after feeding prebiotic substances has been reported before showing a diverse effect of these substances.

At the same time expression of BAX tended to be increased in ileum of animals of both experimental feeding groups. This may be caused by one of the fermentation products. Butyrate has been proven to increase apoptosis by up-regulation of BAX expression (Mandal et al., 2001). An increase of apoptosis has also been reported for inulin itself and was suggested to be an anti-carcinogenic effect (Femia et al., 2002;

Hughes and Rowland, 2001). For lactulose no effects on BAX expression were visible in a calf feeding study (Fleige et al., 2007b).

Again inulin seems to have stronger effects on the investigated parameters, but for expression of BAX only a week statistical confirmation exists. Nevertheless both results, decrease of MKI67 and increase of BAX would hint to a possible protective effect against colonic tumours. These may be no problem in calf rearing but to a certain extent those results can be transferred to other species such as pets and finally to the human.

CONCLUSION

The present study was conducted to elicit possible beneficial effects of substances that were reported to have prebiotic activities on livestock animals.

Pollen of *P. massoniana* has been found to have prebiotic properties in foregoing feeding experiments with rats and piglets. Here additional modes of action besides their proposed beneficial effect on gut flora should be investigated. To do this, porcine ileal cell cultures were treated with sterile filtered pollen extracts to remove every possibility of bacterial or pollen wall influences. And indeed pine pollen substances have been found to have lowering effects on cell proliferation and at least a part of these effects can be explained by changes in mRNA expression of CCNA2. Additionally pro-inflammatory effects of pollen compounds have been found when regarding expression changes of IL6 and IL8, that seems to contradict a beneficial effect of Chinese pine pollen on health. But one has to keep in mind that a cell culture experiment with purified pollen extracts allows only a limited view on actions of whole pine pollen *in vivo*. In the gut overall beneficial properties of fermentation products deriving from pine pollen and their effects on intestinal flora may overrule pro-inflammatory signals triggered by a subset of pollen compounds. Nevertheless a proliferation decreasing action of pollen compounds could hint to a possible anti-tumourigenic action of these substances that may be of interest. A closer look to identify effective substances and a subsequent determination of their action *in vivo* would be a desirable continuation of research work on an important preparation of the traditional Chinese medicine.

In the following two other substances with known prebiotic character – inulin and lactulose – were investigated for their effects on livestock performance and health. For these only little data were available for calves, that go further than only performance results (see figures 3 and 4), so the need for a more detailed research was given. The results of the present study show some similar activities of inulin and lactulose but also a whole range of diverse effects of the two prebiotics with regard to animal performance, haematology, intestinal morphology, and mRNA expression of some genes of interest. Inulin showed an improved animal performance with better daily weight gains and feed efficiency with a simultaneous decrease in intestinal epithelial cell proliferation leading to smaller villi and such less absorbing surface in the small intestine. This is even more interesting since signs for increased iron

absorption exist in the form of higher haemoglobin concentrations and haematocrit values. An effect on inflammatory status in the intestine is mediated via an increased expression of anti-inflammatory IL10 in ileum rather than a decreased expression of pro-inflammatory mediators. In all these cases lactulose shows opposite effects with a decreased animal performance in spite of more cell proliferation and longer villi in the small intestine. Effects on inflammatory markers in the gut are mediated by a decrease of TNF expression in jejunum while IL10 is not influenced. These differences have to be due to specific properties of the two prebiotics for example fermentation kinetics or product profile of promoted bacteria species.

On the whole, inulin clearly has been shown to have beneficial effects on calf health and animal performance. Lactulose seems to have some adverse effects on the animals' appetite that may diminish potential positive effects on growth. But due to its anti-inflammatory effects in the intestine it at least may be supportive during intestinal irritations or may have preventive activities against such disturbances, as well as inulin. But possible effects of inulin on iron absorption in veal calves seem to be far more interesting since iron deficiency and subsequent health effects are a severe problem in animal care. The results of the present study have clearly enlarged the knowledge on the action of inulin on calf health (figure 16). Simultaneously the lactulose results at least in part contradict another calf study (figure 17). These discrepancies may in part be caused by a synbiotic feeding in the first study compared to a solely prebiotic feeding in the present. So in future research possible cross reaction effects with probiotic bacteria strains that are often added to milk replacers need to be investigated. Finally it has to be considered if all those effects on animal performance justify additional expenditures on animal feed. This has to be investigated in calf rearing praxis with according cost-benefit calculations.

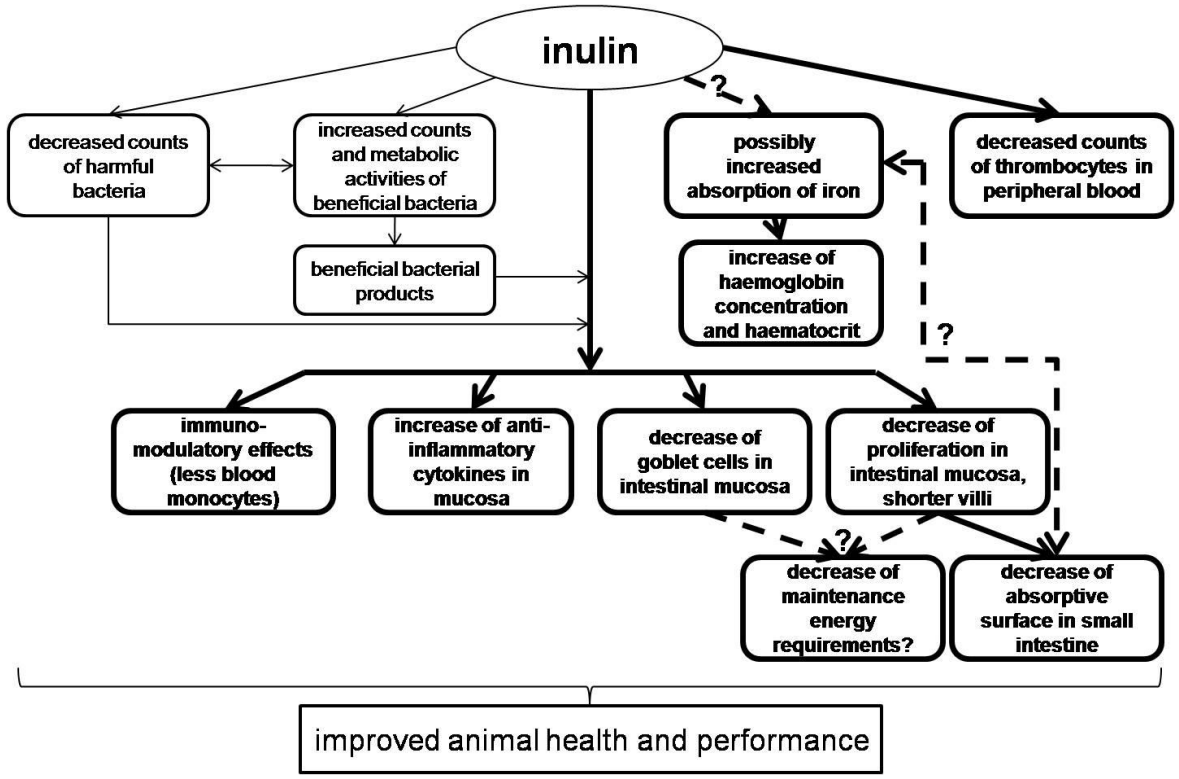

Figure 16: *Current knowledge on the effects of inulin on calf health and performance. Novel findings are highlighted in bold script and arrows. Question marks show assumed interactions that need further proof.*

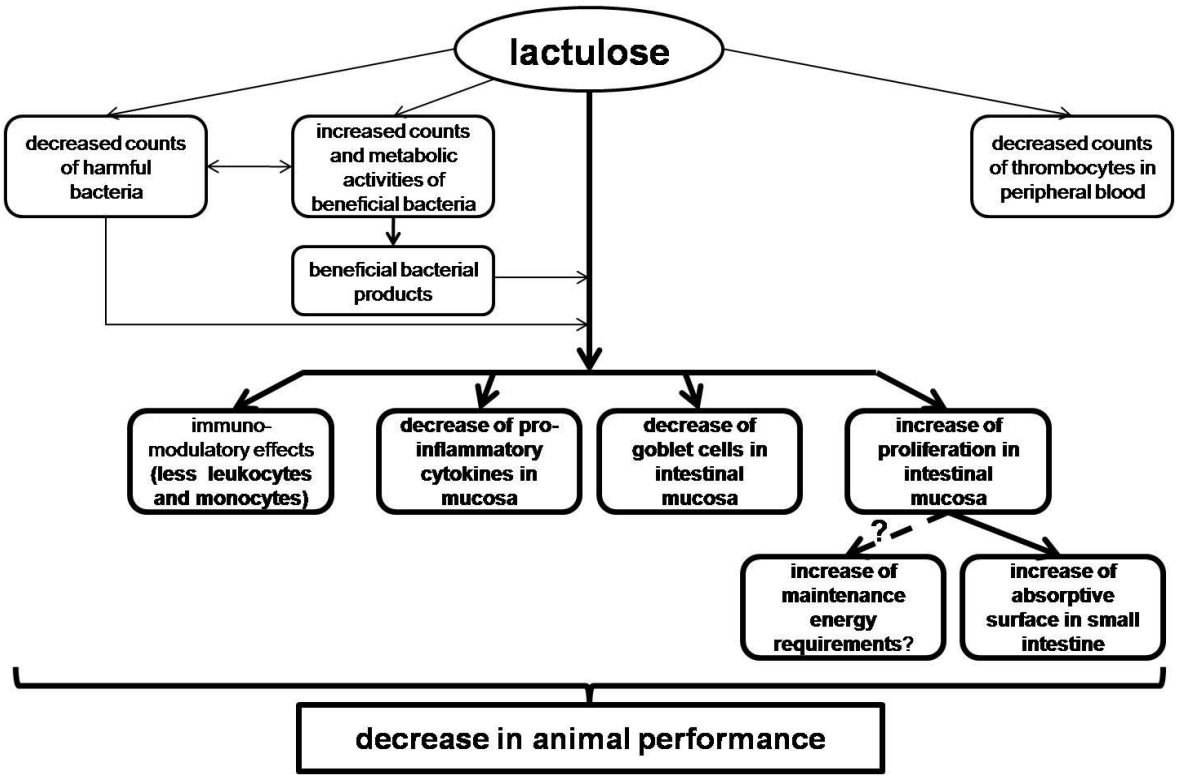

Figure 17: *Current knowledge on the effects of lactulose on calf health and performance. Novel findings – partly contradicting previous results – are highlighted in bold script and arrows. Question marks show assumed interactions that need further proof.*

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SCIENTIFIC COMMUNICATIONS

Original Publications

Masanetz S., Kaufmann C., Letzel T., Pfaffl M.W. (2009):
Effects of pine pollen extracts on the proliferation and mRNA expression of porcine ileal cell cultures.
Journal of Applied Botany and Food Quality 83: 14-18

Masanetz S., Wimmer N., Plitzner C., Limbeck E., Preißinger W., Pfaffl M.W. (2010):
Effects of inulin and lactulose on the intestinal morphology of calves.
Animal 4: 739-744

Masanetz S., Preißinger W., Meyer H.H.D., Pfaffl W.W. (2010):
Effects of the prebiotics inulin and lactulose on the intestinal immunology and hematology of preruminant calves.
Animal (submitted)

Markus W., Hruz T., Docquier M., Pfaffl M.W., Masanetz S., Borghi L., Verbrugghe P., Kalaydjieva L., Bleuler S., Laule O., Descombes P., Gruissem W., Zimmermann P. (2010):
RefGenes: an online tool to identify reliable and condition specific reference genes for quantitative RT-PCR.
Nucleic Acids Research (submitted)

Scientific Presentations

Masanetz S:
Effects of Masson pine pollen extracts on cell vitality.
1st ECIS Research Meeting, 30.-31.07.2009, Regensburg

Posters

Masanetz S, Schedle K, Windisch W, Pfaffl MW:
Effekte von Pinienpollenextrakten auf das Wachstum von ilealen Schweinezellkulturen.
6. BOKU Symposium Tierernährung, 15.11.2007, Vienna, Austria

Masanetz S, Kaufmann C, Letzel T, Pfaffl MW:
Effects of Masson pine pollen extracts on the gene expression profile of porcine ileal cell cultures.
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Masanetz S, Kaufmann C, Letzel T, Pfaffl MW:
Effects of pine pollen extracts on the proliferation of porcine ileal cell cultures.
44. Vortragstagung der DGQ, 16.-17.03.2009, Freising

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APPENDIX

International reviewed publications of the author

Appendix I: Masanetz S, Kaufmann C, Letzel T, Pfaffl MW (2009):
Effects of pine pollen extracts on the proliferation and mRNA expression of porcine ileal cell cultures.
Journal of Applied Botany and Food Quality 83: 14-18

Appendix II: Masanetz S, Wimmer N, Plitzner C, Limbeck E, Preißinger W, Pfaffl MW (2010):
Effects of inulin and lactulose on the intestinal morphology of calves
Animal 4: 739-744

Papers in preparation of the author

Appendix III: Masanetz S, Preißinger W, Meyer HHD, Pfaffl MW (2010):
Effects of the prebiotics inulin and lactulose on intestinal immunology and hematology of preruminant calves.
Animal: Submitted

Papers as co-author:

Appendix IV: Markus W., Hruz T., Docquier M., Pfaffl M.W., Masanetz S., Borghi L., Verbrugghe P., Kalaydjieva L., Bleuler S., Laule O., Descombes P., Gruissem W., Zimmermann P. (2010):
RefGenes: an online tool to identify reliable and condition specific reference genes for quantitative RT-PCR.
Nucleic Acids Research: Submitted

Appendix I

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Effects of pine pollen extracts on the proliferation and mRNA expression of porcine ileal cell cultures.

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Effects of pine pollen extracts on the proliferation and mRNA expression of porcine ileal cell cultures

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Summary

Masson pine pollen has been used in the traditional Chinese medicine for several hundred years and is attributed a variety of health promoting effects including effectiveness against disorders of the digestive system. But only in recent years some evidence has been found that pine pollen and its compounds do influence e.g. inflammatory activities in mice or mRNA expression profiles in piglets. At least a part of these effects have been attributed to the content of polyphenols in pollen.

In the present study different extracts of *Pinus massoniana* pollen were analyzed for their effects on cell proliferation and mRNA expression levels of selected genes. Cell proliferation was analyzed using an electronic cell impedance sensing technique and relative gene expression profiles were investigated using qRT-PCR. It was found that water and 50% ethanol extracts of Masson pine pollen at a concentration equivalent to 1% unprocessed pollen decreased cell proliferation significantly ($p<0.05$ to $p<0.01$). A 100% ethanol extract only transiently delayed cell growth ($p<0.05$ to $p<0.001$). 80% methanol and hexane extracts had no effects on cell proliferation. At the same concentration only the 50% ethanol extract led to a significant up-regulation of the relative expression levels of the pro-inflammatory genes IL-6 and IL-8 and to a down-regulation of proliferation promoter cyclin A ($p<0.05$).

LC-ESI-ToF-MS was performed to get a first impression of the compounds that may be responsible for the effects observed. Distinct mass signals have been identified that can be found in the effective pollen extracts but not in 80% methanol or hexane extracts. A further identification of additional substances could not be performed yet.

Introduction

Pollen of the Chinese yellow pine – *Pinus massoniana* – has been used in traditional Chinese medicine for a long time for its health supporting effects or to directly treat a variety of diseases including disorders of the digestive system (Choi, 2007). In modern times pine pollen is also used in the food and cosmetic industries and its health promoting effects are advertised worldwide. But up until now only few studies have been performed to verify those claims. It has been shown that Masson pine pollen consists mostly of cell wall components delivering only little metabolisable energy (Zhao et al., 1996). General studies of pine pollen found different carbohydrates (Bouveng, 1963), fatty acids (Scott and Strohl, 1962) and finally a pattern of polyphenolic substances (Strohl and Seikel, 1965). Especially the latter are commonly known for their anti-oxidative, anti-carcinogenic and immuno-stimulatory effects (Lambert et al., 2005; Sehm et al., 2006). A more recent study found that addition of Masson pine pollen to the feed modified mRNA expression levels of inflammatory, cell cycle and growth associated genes in colon of piglets (Schedle et al., 2008). Choi (2007) reported that *Pinus densiflora* pollen extracts exhibited anti-nociceptive and anti-inflammatory activities in mice. These effects were attributed to the content of polyphenolic compounds in the pollen. Similar extracts of *Pinus densiflora* pollen were found by Lee et al. (2008) to have strong anti-oxidative and anti-inflammatory activities.

The present study was performed to investigate the effects of different extracts obtained from pollen of *Pinus massoniana* on cell proliferation and expression profiles of selected genes in a porcine ileal cell culture (IPI-21). Furthermore a first attempt was made to identify pollen compounds responsible for effects observed during this work.

Material and methods

Pollen extracts

Masson pine pollen was shock frozen with liquid nitrogen prior to breaking with a ball mill to prevent thermal damage to compounds. Subsequently 10 g of ground Masson pine pollen were extracted with 60 ml of one of the following solvents: a) water, b) 50% ethanol, c) 100% ethanol or d) a mixture of hexane and 80% methanol (v/v=1:1). Extraction was performed at room temperature for 24 h. Hexane and 80% methanol formed two distinct phases which were further processed individually. Extracts were dried using either vacuum centrifugation or lyophilization. Remaining residues were weighed and were then dissolved in phosphate buffered saline (PBS) containing 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich, Steinheim, Germany) to enhance solubility of lipophilic substances. Extracts were sterile filtered (Minisart 0.2 µm, Sartorius AG, Göttingen, Germany) and stored at -20°C. For control treatments extracts were produced with the same solvents but without addition of pollen.

Cell proliferation

Cell proliferation of the porcine ileal cell line IPI-21 was investigated with an electric cell-substrate impedance sensing device (ECIS™ Model 1600, Applied Biophysics, Troy, New York). Cells were cultured at a density of $2.0x10^4$ cells/well in 8-well arrays (ECIS Cultureware 8W10E, Applied Biophysics) fitted with 10 electrodes per well. Extracts were added to reach a final concentration equivalent to 1% (w/v) whole pollen in the medium with a total volume of 300 µl medium. Control treatments were performed with equal amounts of corresponding control extracts. Impedance was measured every three minutes at 30 kHz. Impedance results for each well were normalized to the first value obtained immediately after addition of cells and before cells were attached.

mRNA expression

After 48 h of cultivation total RNA was extracted using Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA concentrations were measured with a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) and diluted to a working solution of 10 ng/µl. RNA quality was checked to be sufficient with a Bioanalyzer 2100 (Agilent, Palo Alto, CA). qRT-PCR reactions were performed with Rotor-Gene 3000 (Corbett

Life Science, Sydney, Australia) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). Specific primer sets for all investigated genes were designed using primer3 v. 0.4.0 (http://frodo.wi.mit.edu/) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Forward and reverse primer sequences are given in Tab. 1. The run protocol consisted of reverse transcription (10 min at 50°C), denaturation (5 min at 95°C) and an amplification and quantification step consisting of 45 cycles (15 s at 95°C, 30 s at 60°C, 20 s at 68°C).

Pollen compounds

Liquid chromatography coupled by electrospray ionisation to time-of-flight mass spectrometry (LC-ESI-ToF-MS) was applied to achieve detailed information of compounds contained in the different extracts. The separation was performed with a Prontosil 120-3-C18 reversed-phase column (Bischoff Chromatography, Leonberg, Germany). After spraying the compounds via an electrospray the screening analysis was performed by simultaneous detection of positively and negatively charged ions with a high-resolution and high-accuracy time-of-flight mass spectrometer (6210 ToF LC/MS, Agilent, Santa Clara, CA). Further details can be found in a recently published study using a similar analytical set-up (Grosse and Letzel, 2007).

Statistical analysis

Statistical analysis of cell culture results was done with Student's t-test for each time point. Relative quantification and statistical analysis of gene expression results were performed with REST 2008 V.2.0.1 (Technische Universität München, Corbett Life Science). In both cases four controls were compared to four treated cell culture wells. A significant difference between pollen and control extract treatment was declared at values of $p<0.05$.

Results

Extraction yields

Treatment of the pollen with a ball mill resulted in nearly totally destroyed pollen structures (Fig. 1). Such good accessibility to pollen

Tab. 1: Primer pairs for quantitative RT-PCR

gene	primer sequence		RT-PCR product size
histon H3	for	ACTGGCTACAAAAGCCGCTC	232 bp
(reference)	rev	ACTTGCCTCCTGCAAAGCAC	
ubiquitin	for	AGATCCAGGATAAGGAAGGCAT	198 bp
(reference)	rev	GCTCCACCTCCAGGGTGAT	
GAPDH	for	AGCAATGCCTCCTGTACCAC	187 bp
(reference)	rev	AAGCAGGGATGATGTTCTGG	
caspase 3	for	TGTGTGCTTCTAAGCCATGG	158 bp
(pro-apoptotic)	rev	AGTTCTGTGCCTCGGCAG	
cyclin A	for	GCAGCAGCCTTTCATTTAGC	116 bp
(proliferation)	rev	TGAAGGTCCAGGAGACAAGG	
TGFβ1	for	TACTACGCCAAGGAGGTCAC	155 bp
(anti-inflammatory)	rev	TCTGCCCGAGAGAGCAATACA	
IL-6	for	AAGGTGATGCCACCTCAGAC	151 bp
(pro-inflammatory)	rev	TCTGCCAGTACCTCCTTGCT	
IL-8	for	GGCAGTTTTCCTGCTTTCTGC	153 bp
(pro-inflammatory)	rev	CAGTGGGGTCCACTCTCAAT	

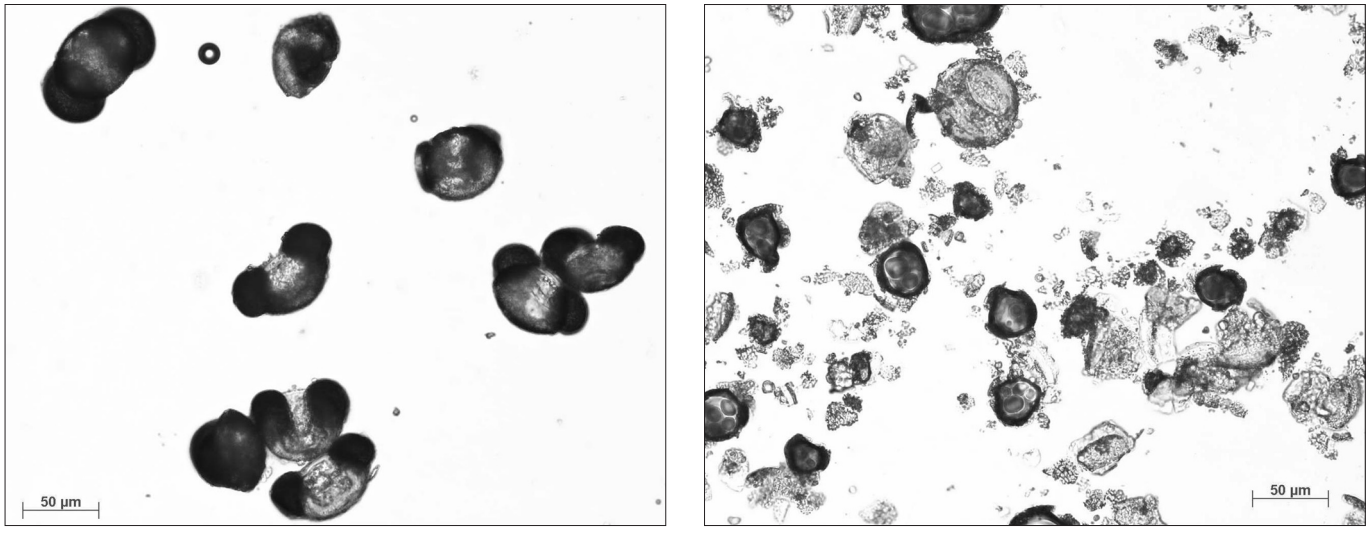

Fig. 1: Masson Pine pollen before **(A)** and after **(B)** breaking with a ball mill.

compounds for extraction should be guaranteed.
The highest extraction yields with 18.3% and 13.7% of total pollen dry matter were achieved with water and 50% ethanol respectively. 100% ethanol extracted 5.8% of pollen compounds. Hexane (6.1%) and 80% methanol (2.3%) in combination extracted 8.4% of pollen dry matter. Control extractions did not result in measurable quantities.

Cell proliferation

Investigation of cell proliferation with the ECIS device showed a high reproducibility with comparatively little variance between single growth curves within one setup. For a better graphic representation standard errors of the mean are only shown for data points every two hours.
Comparison of growth curves revealed that water (data not shown) and 50% ethanol extracts (Fig. 2) of Masson pine pollen decreased impedance values significantly to 70% ($p<0.05$) and 50% ($p<0.05$ to $p<0.01$) respectively of the values of control treated wells. Significant differences were visible after approximately 15 to 20 h after seeding. 100% ethanol pollen extract (data not shown) only transiently decreased cell densities between eight and 24 h after seeding ($p<0.05$ to $p<0.001$) to 80-90% of the control treatment. 80% methanol (data not shown) and hexane extracts (Fig. 3) had no significant effects on cell proliferation.

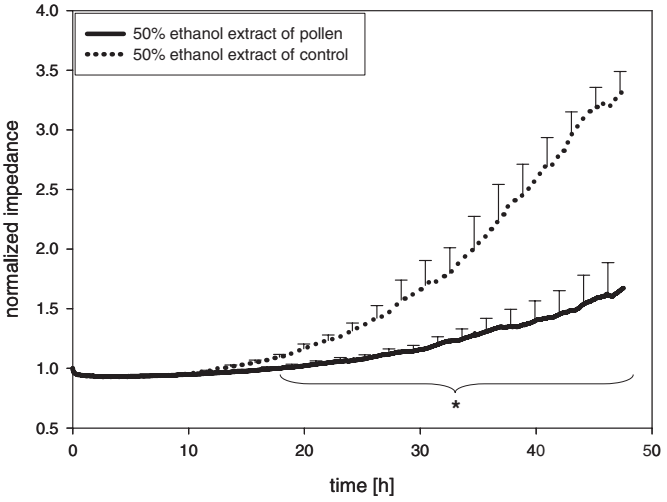

Fig. 2: Water extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium decreased normalized impedance values representing cell densities significantly from ca. 18 h after seeding when compared to cells treated with control extracts without pollen compounds. Data is represented as mean + standard error of the mean (every two hours). Asterisk shows significant differences ($p < 0.05$).

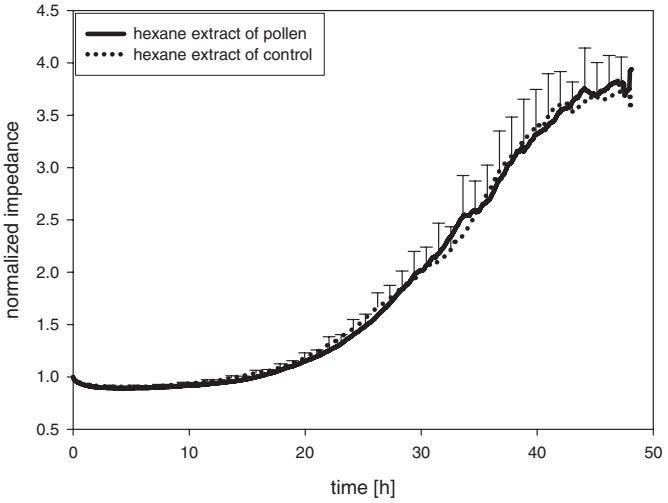

Fig. 3: Hexane extracts of Masson pine pollen at a concentration equivalent to 1% whole pollen in the medium had no significant effects on cell growth when compared to cells treated with control extracts without pollen compounds. Data is represented as mean + standard error of the mean (every two hours).

mRNA expression

Of all pollen extracts only the 50% ethanol extract (Fig. 4) led to a significant up-regulation of mRNA expression levels of the pro-inflammatory genes IL-6 (factor 4.3, $p=0.012$) and IL-8 (factor 2.3, $p=0.024$) and to a down-regulation of the proliferation regulator cyclin A (factor 0.7, $p=0.039$). All other extracts did not have significant effects on mRNA expressions (data not shown).

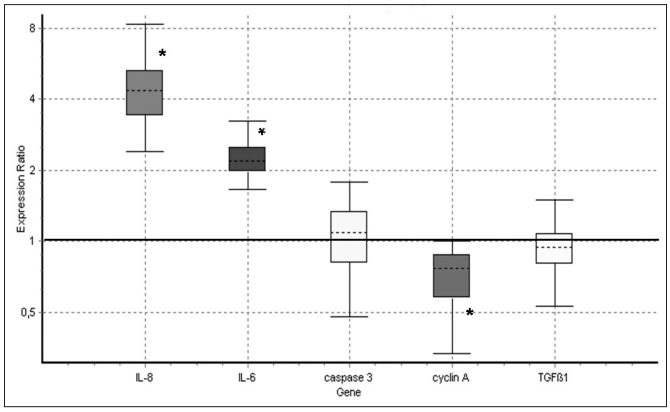

Fig. 4: 50% ethanol extract of Masson pine pollen significantly increased relative expression of IL-8 and IL-6 and decreased the relative expression of cyclin A compared to cells treated with control extracts without pollen compounds (*: $p < 0.05$)

Pollen compounds

LC-ESI-ToF-MS was performed in order to characterize the compounds responsible for the effects observed. Thereby a number of distinct mass signals has been identified that can be found exclusively in the three pollen extracts that exhibited effects on cell proliferation and/or gene expression (Tab. 2). These signals were not detected in both 80% methanol and hexane extracts of Masson pine pollen. A detailed identification of these substances was not yet possible due to missing reference substances. However, most compounds could be characterized via hydrophobicity (retention factor), ionization behavior and the molecular formula. A database containing the three independent parameters (BERKEMEYER and LETZEL, 2008) will identify these compounds very specific in forthcoming samples.

Discussion

Traditional Chinese medicine and its products are a growing market in the western countries. Masson pine pollen is one of these products heavily advertised in internet stores for its health promoting effects. The present study aimed to verify some of these claims. Therefore

Tab. 2: LC-ESI-MS signals specific for effective Masson pine pollen extracts.

	water extract	50% ethanol extract	100% ethanol extract
m/z (-)		135.050	
	146.107	146.107	
	204.094	204.094	
	230.167		
	328.231	328.230	
	330.235		330.199
m/z (+)		129.086	
	131.101		
	135.053		
	146.104		
	235.126	235.126	235.111
		237.097	
	254.127		
		318.217	
		334.218	

a combination of a real-time cell monitoring system, qRT-PCR and LC-ESI-ToF-MS was used for the first time.
Especially the ECIS system enables to monitor changes in impedance values of a cell culture at very short intervals with a high reproducibility. Such it was possible to compare cell growth of four repetitions of each pollen extract and mock treated culture wells with 960 single t-tests – every three minutes for 48 h. A resolution that was not possible with conventional cell culture techniques.

The yields of the extraction methods (up to 18.3% w/w for the water extract) used in this study were relatively similar to the yield obtained by CHOI (2007). Their extraction of intact *Pinus densiflora* pollen with 70% ethanol for three days at room temperature resulted in a yield of 8%. But since in the present study pollen was broken prior to extraction it is to be expected that a broader range of pollen compounds is reached simultaneously.

Since most of the research work on pollen effects is done in feeding experiments there is only little data available on pollen effects on cell growth and gene expression *in vitro*. In a piglet feeding study addition of *Pinus massoniana* pollen resulted in increased villus heights in the small intestine (SCHEDLE et al., 2008). In feeding experiments effects of pine pollen are often attributed to the increase of ingestion of cell wall components (ZHAO et al., 1996). Since non-soluble cell wall constituents should be absent in the pollen extracts it is likely that other pollen compounds trigger the results of the present study. Additionally a decrease in cell proliferation is rather opposed to the increase of villus lengths observed in the piglet study.
The bioactive properties of bee pollen extracts (KROYER and HEGEDUS, 2001) as well as the anti-nociceptive and anti-inflammatory properties of extracts from *Pinus densiflora* pollen (CHOI, 2007) have been attributed to the high content of polyphenolic substances. Since polyphenols of different plants have been found to decrease cell proliferation in different cell lines (e.g. KANEUCHI et al., 2003; BRIVIBA et al., 2002; IIJIMA et al., 2000) such compounds of the Masson pine pollen could be an explanation for the results observed in the present study. Different types of polyphenols also may show different solubilities in lipophilic and hydrophilic solvents so it is possible that not all extracts lead to similar decreases of cell proliferation.
Water and 50% ethanol extracts revealed similar effects on cell proliferation. But only substances in the 50% ethanol extract appeared to influence mRNA expression of cyclin A as a possible explanation for the decrease of proliferation observed. It is to be expected that additional regulators of cell proliferation or cell death are also targeted by water soluble substances that may also be present in the 50% ethanol extract. A similar regulation of cyclin A has been found before for red wine polyphenols (IIJIMA et al., 2000).
A modulation of the pro-inflammatory IL-6 and IL-8 suggests that pollen compounds present in the 50% ethanol extract are able to alter the fine tuning of the inflammatory status of ileal cells. The regulations found in the present study hint to an increase of the inflammatory status of the cultured cells. In contrast to this polyphenols mostly have been found to have anti-inflammatory properties. Nevertheless there is evidence for some polyphenolic substances – e.g. *cis*-resveratrol – to increase expression of pro-inflammatory interleukins such as IL-6 (LEIRO et al., 2005). But in contrary to the findings of the present study 70% ethanol extracts of *Pinus densiflora* pollen have been found to inhibit the production of pro-inflammatory signal molecules – including IL-6 – in macrophages after activation with LPS (LEE et al., 2009).
Unfortunately none of the pollen compounds could be identified with the methods available for the present study. Nevertheless it was possible to at least identify mass signals of compounds exclusively found in the water, 50% ethanol and 100% ethanol extracts. Such one or a combination of some of these may be responsible for the effects observed with those extracts.

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Appendix II

Masanetz S, Wimmer N, Plitzner C, Limbeck E, Preißinger W, Pfaffl MW (2010):
Effects of inulin and lactulose on the intestinal morphology of calves
Animal 4:5: 739-744

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Effects of inulin and lactulose on the intestinal morphology of calves

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For some time now prebiotics have been proposed to improve health by stimulation of beneficial bacteria in the intestine of humans and animals. The current study is aiming to show effects of feeding of either 2% inulin or 2% lactulose in milk replacer on performance and intestinal morphology of male Holstein–Friesian calves. After 20 weeks of feeding inulin led to significantly higher daily weight gains than lactulose while control animals ranged between the experimental feedings. Ingestion of milk replacer was reduced in lactulose treated animals. Additionally differences of villus height in jejunum ($P = 0.07$) and ileum ($P = 0.03$) could be found with an increase for lactulose treated animals and a decrease for inulin treated animals. In ileum the density of proliferative epithelial cells tended to be lower in inulin treated and higher in lactulose treated animals ($P = 0.08$). Both inulin and lactulose tended to decrease the quantity of goblet cells in the tips of ileal villi ($P = 0.07$). Both prebiotics can affect performance and intestinal morphology of calves and may as such affect animal health. But effects differ between substances.

Keywords: calves, intestine, inulin, lactulose, morphology, prebiotics

Implications

Research on prebiotic substances has been done mostly on human subjects or small laboratory animals. And mostly only changes in gut bacteria populations were investigated disregarding effects on the intestine itself. Since prebiotics are claimed to be a possible alternative to antibiotics as growth promoters in livestock husbandry this study has been done on calves to further investigate potential beneficial effects on cattle health. To determine modes of action of the prebiotics inulin and lactulose changes of morphology in the small and large intestine and the adjoining immune defense tissues have been observed.

Introduction

In recent years the European Union introduced a ban on antibiotics as growth promoters in animal feed. Now alternatives have to be found to assure animal health and performance during meat or milk production. Possible beneficial feed additives may be prebiotics such as inulin or lactulose which can be able to modulate intestinal bacterial populations towards a healthier flora (Gibson and Roberfroid, 1995).

Inulin is a natural constituent of a wide range of plants including many common vegetables and cereals in western diet (Van Loo *et al.*, 1995). It consists of β-(2-1)-linked fructo-oligosaccharides of varying degrees of polymerization from 2 to 60 sugar units. In the intestine inulin leads to a shift in the bacterial flora towards more bifidobacteria regarded as beneficial for host health (Gibson *et al.*, 1995). Additionally inulin-type fructans have been shown to have anti-carcinogenic properties (Hughes and Rowland, 2001; Femia *et al.*, 2002). Fructo-oligosaccharides were also reported to enhance the performance of livestock including pigs and calves (reviewed by Van Loo, 2007).

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a semi-synthetically produced disaccharide that does not occur naturally (Schumann, 2002). It is fermented by lactobacilli and bifidobacteria, but also by other bacteria species such as *Clostridium perfringens*, *Escherichia coli* or *Bacteroides* spp. (Mitsuoka *et al.*, 1987). Lactulose is commonly used to treat constipation (Attar *et al.*, 1999) and hepatic encephalopathy (Bircher *et al.*, 1966) but also to enhance the animal performance (Fleige *et al.*, 2007).

Until now most of the studies were done on human subjects or laboratory rats instead of livestock and mostly

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studied changes in bacterial flora. Comparably little research has been done on possible changes of intestinal morphology. Thus the object of this study was to provide information about the influences of inulin and lactulose feeding on performance, morphology of the intestinal mucosa and on mesenteric lymphoid nodes in pre-ruminant calves.

Material and methods

Animals, husbandry, feeding and experimental procedures

Forty-two male Holstein–Friesian calves were purchased from the Viehzentrum Waldkraiburg GmbH and housed at the experimental station Karolinenfeld (Bayerische Landesanstalt für Landwirtschaft – LfL, Institut für Tierernährung und Futterwirtschaft). The animals were subdivided into three experimental groups ($n = 14$ per group) with balanced weight (52.9 ± 6.2 kg) and age (22 ± 5 days).

Compositions of the three diets are given in Table 1. Calves of the control group were fed with the milk replacer Milkibeef Top (Milkivit, Trouw Nutrition, Burgheim, Germany). The other groups were fed with the same milk replacer iso-energetically and iso-nitrogenically enriched with either 2% inulin (Beneo® ST, Orafti, Tienen, Belgium) or 2% lactulose (Lactusat, Milei GmbH, Leutkirch, Germany). Calves were fed individually by transponder automatic feeders (Förster Technik, Engen, Germany). During the experimental period the milk replacer concentration was rising from 125 g/l to 200 g/l with daily intake volumes rising from 6 l to 16 l. Calves had free group access to fresh drinking water and up to 300 g hay per day and animal. Since the calves were housed on straw a further uptake of roughage could not be excluded. The animals were slaughtered after 20 weeks.

Histological sampling

Immediately after slaughtering the gastrointestinal tract was removed from the carcass. Sections of 0.5 to 1 cm of centre parts of jejunum, ileum and colon as well as mesenteric lymphoid nodes were collected. Tissue samples were washed in physiological NaCl solution and placed in neutral buffered of 3.7% formalin (Carl Roth GmbH, Karlsruhe, Germany) for 24 h. At the Landesuntersuchungsamt (LUA, Oberschleißheim) samples where embedded in paraffin. Sections of 4 to 6 μm were cut (Microtom LEICA RM2145, Leica, Wetzlar, Germany), deparaffinized and rehydrated before further treatment. All microscopic analyses were done randomly by one person without knowledge of treatment groups. Histological sections of ileum, jejunum and mesenteric lymphoid nodes were examined with the light microscope Axioskop 2 plus (Zeiss, Oberkochen, Germany), sections of the colon with the stereomicroscope Stemi 2000-C (Zeiss). Pictures were taken with the AxioCam MRc (Zeiss) and analyzed with the connected software AxioVision 3.1.

Histomorphometry

Histomorphometry was done on sections stained with Alcian blue/periodic acid Schiff's reagent (AB-PAS) (see histochemistry) or haematoxylin (Carl Roth GmbH, Karlsruhe, Germany) and eosin yellowish solution (Fluka-Chemie AG, Buchs, Switzerland). Measurement techniques were adapted from Sehm *et al*. (2006). Briefly villus length from lamina muscularis to tip and width were measured in sections of jejunum and ileum. Crypt depth from lamina muscularis to the crypt mouth and distance between crypts were examined in sections of the colon. Figure 1 gives an overview of conducted measurements on the intestinal mucosa. All were done on three well-defined villi or crypts of one section. In mesenteric lymphoid nodes and in ileal Peyer's patches the number of lymph follicles in a defined area and areas of these follicles were measured.

Histochemistry

The AB-PAS staining method was used to investigate the number of goblet cells in jejunum and ileum. Briefly sections

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

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

DM = dry matter.

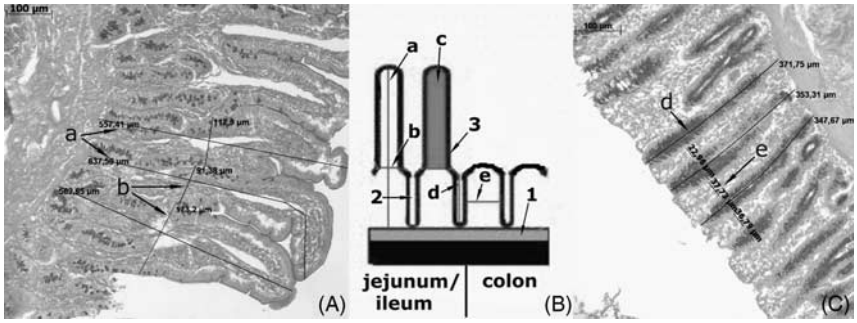

Figure 1 Morphological measurements in the small and large intestine*:* Conducted measurements are shown as an overview for both small and large intestine (B) and as actual measurements in sections of jejunum (A) and colon (C). (1) *lamina muscularis mucosae*, (2) crypt of Lieberkühn, (3) villus; small intestine: (a) villus length, (b) villus width, (c) villus tip; large intestine (no villi existent), (d) crypt depth, (e) distance between crypts.

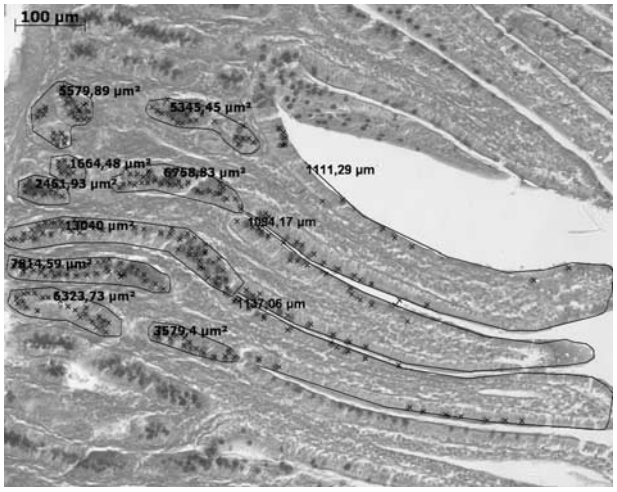

Figure 2 Quantitative analysis of goblet cells: goblet cell density was determined as number of goblet cells (x) per length of villus or crypt outline.

pre-treated with 3% acetic acid were treated with 1% Alcian blue in 3% acetic acid and subsequently with 0.5% periodic acid. Tissues were then treated with Schiff's reagent (all chemicals from Carl Roth GmbH, Karlsruhe, Germany) and counterstained with haematoxylin. AB-PAS positive cells were counted individually for crypts and villi of jejunum and ileum. Goblet cell density was determined as number of AB-PAS positive cells per length of villus tip or crypt outline (Figure 2). Quantification of goblet cells in the colon was not possible due to the high density of AB-PAS stained cells in this area.

Immunohistochemistry

Detection of proliferative cells was done on six randomly selected samples of each treatment group with the antibody MIB-1 (Dako cytomation, Glostrup, Denmark) directed against the proliferation marker Ki-67. For antigen accessibility sections were incubated in boiling 0.01 M citrate buffer (pH 6) and all washing was done in phosphate buffered saline with 0.05% Tween (phosphate buffer saline (PBS)-T). To block endogenous peroxidase activity and unspecific antibody binding the slides were treated with 1% hydrogen peroxide and 10% goat serum (Dako cytomation), respectively. Binding of MIB-1 (1 : 50 in PBS) was done at 4°C over night. Afterwards sections were incubated with horse raddish peroxidase-labeled polyclonal goat anti-mouse antibody (1 : 50 in PBS, Dako cytomation). Visualization was done with 3,3′-diaminobenzidine solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Tissue was then counterstained with haematoxylin. Quantification of MIB-1 positive cells was done similarly to AB-PAS positive cells in the crypts of jejunum, ileum and colon.

Statistical analysis

For each parameter the mean group values and the residual standard error of the mean were determined. Comparison of dietary treatments was done with the one-way ANOVA method of SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). Homoscedascity among compared groups was not formally tested as the test would pose a large risk of type II statistical error due to limited sample size (Altman, 1985). However, the data was screened using a scatter plot. The individual contrasts were tested using the conventional Tukey–Kramer test for multiple comparisons. Nutritional effects were considered if $P < 0.05$. Tendency was considered if $P < 0.1$.

Results

During the current experiment general animal performance data also were collected. Animals fed inulin had significantly ($P < 0.05$) higher daily weight gains (1269 $\pm$ 101 g/day) than animals fed lactulose (1124 $\pm$ 178 g/day) while the control treatment led to intermediate values (1203 $\pm$ 152 g/day). Daily ingestion of milk replacer (dry matter) was very similar between inulin and control groups (1702 $\pm$ 128 g/day and 1709 $\pm$ 117 g/day, respectively) while calves of the lactulose group collected significantly less ($P < 0.05$) of their daily allowance (1581 $\pm$ 181 g/day). Since hay consumption was not determined for each individual animal, feed efficiency was not statistically analyzed. Calculating with group averages total feed efficiency (average dry matter intake/daily gain) was slightly better for inulin (1.55) than for control (1.64) or lactulose (1.62). All data has been reported previously by Preißinger *et al.* (2007) (Table 2).

Table 2 *Results of measurements on mucosal architecture, goblet cell numbers, lymph follicles in Peyer's patches and mesenteric lymphoid nodes (each* n = *14 if possible) and on cell proliferation in intestinal sections* (n = *6) (all values are demonstrated as mean* ± *s.e.m.)*

	Treatment groups				
	Control	Inulin	Lactulose	s.e.m.	*P*-values
Villus height (mm)					
Jejunum	0.77	0.71	0.84	0.144	$P = 0.07$
Ileum	0.69[a]	0.59[a,b]	0.78[b]	0.175	$P = 0.03$
Villus width (mm)					
Jejunum	0.13	0.13	0.12	0.024	ns
Ileum	0.11	0.10	0.12	0.023	ns
Crypt depth (mm)					
Colon	0.42	0.40	0.42	0.078	ns
Distance between crypts (mm)					
Colon	0.02	0.02	0.03	0.008	ns
Goblet cells/mm (villus tip)					
Jejunum	6.2	10.2	8.0	5.71	ns
Ileum	25.9	17.3	15.8	10.62	$P = 0.07$
Goblet cells/mm (crypt)					
Jejunum	28.0	29.5	30.3	11.86	ns
Ileum	70.6	61.5	61.3	13.00	ns
Proliferative cells/mm					
Jejunum	59.7	51.0	62.0	25.69	ns
Ileum	45.9	32.0	59.4	19.31	$P = 0.08$
Colon	26.4	13.1	19.0	11.85	ns
Lymphoid nodes					
Follicle area (mm^2)	0.07	0.07	0.07	0.020	ns
Follicle density (mm^{-2})	1.80	1.88	1.77	0.589	ns
Peyer's patches					
Follicle area (mm^2)	0.08	0.09	0.07	0.038	ns
Follicle density (mm^{-2})	7.04	6.89	7.03	2.379	ns

ns = not significant.
Significant difference between a and b: $P = 0.02$ (Tukey-Kramer test).

In jejunum villus length showed an overall tendency to be decreased in inulin treated animals and to be increased in lactulose treated animals when compared to the control group ($P = 0.07$). A very similar but statistically significant difference could be found for villus length in the ileum ($P = 0.03$). Tukey–Kramer test revealed significant differences between the two experimental feeding groups ($P = 0.02$) but not between inulin or lactulose treatment and the control group. No nutritional effects could be seen for villus width in jejunum and ileum or crypt depth and distance between crypts in the colon.

Densities of proliferative epithelial cells tended to be decreased by inulin and to be increased by lactulose feeding in ileum ($P = 0.08$) but not in jejunum and colon.

Goblet cell densities in jejunum and ileum were determined individually for crypts and villus tips. In villus tips of the ileum a tendency for a decrease of goblet cell density ($P = 0.07$) could be found for both experimental treatments. No further significant differences could be detected.

Area and density of lymph follicles were determined in slides of mesenteric lymphoid nodes and ileal Peyer's patches, but no significant differences could be found between the different feeding regimes.

Discussion

Prebiotics are one possible alternative to antimicrobial growth enhancers to improve animal health and performance especially during critical periods such as weaning. Therefore a variety of studies on influences of inulin and oligofructose on animal performance have already been accomplished. Quite a number found no or only little effects, but some reported effects similar to this study with improved daily weight gain and feed efficiency (Kaufhold *et al.*, 2000; Flickinger *et al.*, 2003; Van Loo, 2007). Interestingly a significantly higher daily weight gain or an improved feed conversion ratio as was found in the inulin group is commonly associated with a better intestinal nutrient absorption because of longer villi (Wu *et al.*, 1996; Awad *et al.*, 2008), while in this study small intestinal villi were shortened after inulin feeding. A similar decrease of villus length after addition of inulin to the feed has already been reported in weaning piglets (Pierce *et al.*, 2005). But also increases of villus length in rats after feeding of soluble chicory extract (1% to 5%) or purified inulin (5%) (Kim, 2002) were found. Supporting the observed shortening of villi a decrease of MIB1-positive and hence considered proliferating cells was found in ileum of calves fed inulin. A comparable lowering of cell proliferation has been reported

before in rats fed inulin and oligofructose at a concentration of 10% of the diet (Femia *et al*., 2002). Concluding it may have to be considered that shorter villi and a decreased proliferation rate may reduce the amount of energy necessary for maintenance of gut architecture thereby providing more energy for growth and fattening. A decrease of goblet cell density in ileal villus tips, also found in the inulin group, may further lower energy requirements for mucus production. In contrast, an increase of goblet cell densities after feeding inulin and other non starch polysaccharides to rats has been detected before (Kim, 2002). However, as a support for this study a significant decrease of goblet cell numbers was found in weaned piglets fed a diet supplemented with 10% carob tree seed meal as a fermentable fiber source (Van Nevel *et al*., 2005).

Although lactulose has also been considered as a prebiotic only comparably little data on animal performance has been available up until now. In contrast, to this results Fleige *et al*. (2007) found a significantly increased feed consumption and a tendency for improved daily weight gain for calves fed 3% lactulose and a probiotic bacteria strain with their milk replacer. Comparable to these findings with inulin they also found a decrease of villus length in ileum of said calves. Directly opposed to these effects lactulose treatment in this study led to lower feed intakes and daily weight gains and simultaneously increased villus heights in the small intestine compared to the control group. Possibly the addition of *Enterococcus faecium* to the milk replacer in the study of Fleige *et al*. (2007) had a modulating influence on lactulose derived effects leading to different regulations of appetite and villus length. In accordance to the longer villi lactulose also tended to increase the number of proliferative cells in ileum. Similar to this finding other dietary fibers such as highly fermentable guar gum and pectin are also known to increase the proliferation zone in the caecum crypts of rats (Brunsgaard and Eggum, 1995). Interestingly the notable decrease of nutrient ingestion ($\sim$10%) of lactulose fed calves was not enough to antagonize the increase of villus length or proliferation rates as may be expected with respect to studies on fasted animals (Clarke, 1975). But, on the other hand, no reduction in villus height was found in pigs fed with a low-energy diet compared to a diet with twice the energy content (Claus *et al*., 2006) which would be more similar to the decrease in energy uptake in the lactulose group than starved animals. An expected improvement of animal performance due to a better intestinal morphology (Wu *et al*., 1996; Awad *et al*., 2008) may be overruled by the less nutrient ingestion observed in the lactulose group. But similar to inulin lactulose also led to a decrease in goblet cell density in villus tips in ileum.

The differences in effects on animal performance and mucosal architecture between the treatment groups are not easily explained by differences in nutrient consumption alone but could be based on partly different fermentation properties of the two prebiotics. It has been found that pre-caecal fermentability of lactulose in pigs was lower than that of inulin (Branner *et al*., 2004) maybe leading to different sites of action in the calves' intestines. Additionally both substances differed in stimulatory effects on beneficial bacterial subpopulations and fermentation product profiles (Rycroft *et al*., 2001). Goblet cell numbers might be regulated by other factors that may be similar between both prebiotics, such as viscosity (Ito *et al*., 2009).

In conclusion this study has shown regulative effects of two commonly used prebiotics on intestinal villus length, proliferation and goblet cell numbers supporting observations already made before in other animals. But the differences in the effects of the two prebiotics on animal performance and intestinal morphology observed in this study raise the question whether prebiotics and their health promoting actions can be generalized. In conclusion the usage of prebiotics would have to be fine tuned for particular purposes raising the need for additional research study.

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Appendix III

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

Animal: Submitted

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

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Abstract

Prebiotics are suggested as alternative to antibiotics in animal rearing. Fermentable substances such as inulin or lactulose have been proposed to stimulate the immune system and health by modulation of the intestinal flora and its fermentation products. In the present study effects of inulin and lactulose on intestinal health and hematology of calves have been investigated. Both prebiotics significantly decreased thrombocyte counts in peripheral blood (P=0.008). Only inulin was able to increase hemoglobin concentration (P=0.003) and hematocrit (P=0.035). Total leukocyte count was decreased by lactulose (P=0.005) while both prebiotics tended to lower monocyte proportions (P=0.073). mRNA expression of inflammation related markers was also affected by the prebiotics. In jejunum tumor necrosis factor alpha expression was decreased by lactulose (P=0.018). In Ileum expression of interleukin 10 was increased by inulin (P=0.048). In colon platelet endothelial cell adhesion molecule-1 was increased by both prebiotics (P=0.023) while lactulose tended to lower interleukin 2 receptor alpha chain expression (P=0.058). mRNA amounts of interleukin 8 were increased by lactulose in mesenteric lymph nodes (P=0.029). Proliferation and apoptosis related genes were affected in ileum. There expression of BCL2-associated X protein tended to be increased by both prebiotics (P=0.096), while the antigen identified by monoclonal antibody Ki67 was increased only by inulin (P=0.009). These results show a clear effect of prebiotics on animal health. Most hint to an anti-inflammatory regulation in the gut due to a possibly decreased intestinal pathogen load. Blood traits support this hypothesis. Additionally inulin treatment shows some effects that may be caused by a support of iron absorption. Thus inulin also may have additional effects on calf health.

Introduction

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

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

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

The present study investigated long term treatment of calves with the prebiotics inulin and lactulose and their potentially beneficial effects on hematology and intestinal health.

Material and methods

Animal husbandry and feeding

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

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

Composition of diets is given in table 1. Basic of all diets was the milk replacer Milkibeef Top (Milkivit, Trouw Nutrition, Burgheim, Germany). Control groups were fed the pure replacer, the other groups were fed the same milk replacer iso-energetically and iso-nitrogenically enriched with 2% (dry matter, DM) of either inulin (Beneo ST, Orafti, Tienen, Belgium) or lactulose (Lactusat, Milei GmbH, Leutkirch, Germany). Individual feeding was achieved by transponder automatic feeders (Förster Technik, Engen, Germany). During the experimental period of 20 weeks the milk replacer concentration was rising from 125 g/L to 200 g/L with daily intake volumes rising from 6 L to 16 L. Calves had free group access to fresh drinking water and up to 300 g hay per day and animal. Since the calves were housed on straw a further uptake of roughage could not be excluded. The animals were slaughtered after 20 weeks.

[Table 1]

Tissue and blood sampling

Ten weeks after the beginning of the feeding experiment and during the slaughtering process after 20 weeks of experimental diet blood samples were taken from the jugular vein for examination by a veterinary laboratory (Vetmed Labor, Unterhaching, Germany). 10 mL of each of the blood samples were also transferred to paxGene Blood RNA tubes (BD, Heidelberg, Germany) for RNA extraction. Tubes were stored at -20°C until extraction. Immediately after slaughtering organs were removed from the carcass. Small pieces

were cut from central parts of jejunum, ileum and colon and from the tip of the spleen. Intestinal samples were washed twice in physiological salt solution to remove digesta. All tissues were immediately flash frozen in liquid nitrogen. Samples were taken in duplicate and were stored at -80°C until extraction to prevent RNA degradation.

RNA extraction and quality control

RNA extraction of whole blood stabilized in paxGene blood RNA tubes was performed using the paxGene blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA extraction of tissue samples was performed with TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA was extracted from small pieces (~50 µg) of tissue. Samples were not allowed to thaw until pieces were immersed in TriFast to avoid RNA degradation.

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

Real-time quantitative reverse transcription PCR (qRT-PCR)

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

[Table 2]

Data Evaluation

Data of hematology examination are presented as mean ± residual standard error of the mean (r.e.m.) with n=14.

For qRT-PCR crossing point values were obtained with Rotor-Gene 6000 software version 1.7 (Corbett Life Science). For relative quantification of mRNA expression a number of potential reference genes were measured and for each tissue the optimal pairs of reference genes were chosen using GenEx Pro Ver 4.3.4 (MultiD Analyses AB, Gothenburg, Sweden). The arithmetic mean of their crossing point values was used to normalize target gene values. Calculation of relative expression values was done using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). For each group results are given in geometric means of expression ratios relative to the control group (=1.0) and r.e.m.. Statistical analysis of gene expression data was done using the one-way ANOVA method of SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). In case of significant differences a post-hoc Tukey-Kramer test was performed to further investigate group differences.

Results

Hematology

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

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

[Table 3]

RNA quality

RNA purity was regarded as good with an average A260/A280 ratio of 2.0±0.01 for all tissues.

Values greater than 1.80 are considered as an indicator for high purity. RNA quality for all tissues was investigated with a Bioanalyzer 2100 (Agilent Technologies) and yielded an average RNA integrity number (RIN) of 6.9±0.13. RIN is a measure for RNA quality ranging from totally degraded RNA (RIN=1) to intact RNA (RIN=10) (Fleige and Pfaffl, 2006; Bustin *et al.*, 2009). A RIN of 5 was regarded as minimal requirement for qRT-PCR. Single samples not sufficient for qRT-PCR were re-extracted and when values did not reach adequate levels were omitted from analysis.

Reference gene pairs

Reference genes are regarded as acceptable if their mRNA expression is stable between treatment groups. GenEx Pro software (MultiD Analyses AB) is one possibility to choose ideal reference gene pairs to normalize mRNA expression data. Here for each tissue the best reference gene pair was chosen from all genes measured (Bustin *et al.*, 2009). Thus normalization was done with actin β (ACTB) and G protein associated kinase (GAK) in jejunum, vacuolar protein sorting 4 homolog A (VPS4A) and GAK in ileum, ubiquitin (UBIQ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in colon, Ras-related protein Rab-21 (RAB21) and peptidase (mitochondrial processing) alpha (PMPCA) in spleen and ACTB and UBIQ in blood.

mRNA expression changes of inflammation and immune modulating factors

An overview of all gene expression changes found in the present study is given in table 4.

In jejunum relative expression of the pro-inflammatory tumor necrosis factor-α (TNF) was influenced by the feeding group (P=0.018). Lactulose induced a down-regulation of its expression compared the control as well as to the inulin group (Tukey-Kramer Test: P=0.025 or P=0.017 respectively).

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

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

In mesenteric lymph nodes expression of the chemo-attractant interleukin 8 (IL8) was significantly up-regulated by lactulose (P=0.029). The up-regulation was significant when compared to control (P=0.027) as well as to inulin (P=0.016).

Interleukin 1β (IL1B), transforming growth factor-β1 (TGFB1), interferon-γ (IFNG), receptor for Fc fragment of IgA (FCAR), CD69, CD4 and CD8b were not influenced by the diet in any tissue analyzed. In spleen and blood no regulations of investigated genes were found (data not shown).

mRNA expression changes of proliferation and apoptosis related genes

Only in ileum changes in expression of proliferation or apoptosis related genes were found. There the proliferation marker antigen identified by monoclonal antibody Ki67 (MKI67) was significantly influenced by both prebiotics (P=0.009). Inulin lead to an up-regulation of its expression relative to the control (P=0.028) while lactulose decreased it slightly. Such its expression was significantly different between inulin and the lactulose group (P=0.003) but not between both and the control. Simultaneously lactulose and inulin showed a weak tendency to increase the relative expression of pro-apoptotic BCL2-associated X protein (BAX; P=0.096).

BCL2-like-1 (BCL2L1), epidermal growth factor receptor (EGFR) and Caspase 3 (CASP3) were not influenced by the diet.

[Table 4]

Discussion

Until now only few studies on prebiotics have been completed with calves. Most of these studies concentrated on animal performance or intestinal bacteria populations (Flickinger *et al.*, 2003). To the authors' knowledge only one other long-time study was performed on calves where changes in gene expression and hematological traits during feeding of lactulose have been investigated. There the authors described an increase of lymphocyte numbers with 1% lactulose and a decrease of thrombocyte numbers in 3% lactulose fed calves (Fleige *et al.*, 2009). The latter finding could be supported by the present study where thrombocyte concentrations were significantly decreased by both prebiotics after ten weeks of feeding. A high rate of thrombocyte aggregation is considered as one of many risk factors for coronary heart disease and has been shown to be reduced by intakes of higher amounts of dietary fiber in rats (Bagger *et al.*, 1996). Maybe decreases in thrombocyte numbers or in their aggregation ratio are one possible explanation for the fact that intake of fiber rich food is inversely correlated with the incidence of coronary heart disease (Pietinen *et al.*, 1996) besides their lowering effects on serum cholesterin (Bagger *et al.*, 1996).

At the end of the experimental period only hemoglobin concentrations and hematocrit were affected by inulin feeding. Ohta *et al.* (1998) reported that these two parameters were significantly reduced after gastrectomy and subsequent malabsorption of iron in rats. But levels could be recovered to those of sham-operated control animals in gastrectomised rats by 0.75% oligofructose feeding. Iron deficiency is a common problem in veal calves fed only milk replacer and no solid feed or fiber rich supplementation. (Cozzi *et al.*, 2002). All calves in the present study also had to be treated with iron supplementation for a short period. Inulin feeding could have had improving effects on iron absorption since evidence exists that it increased the fraction of soluble iron in the caecal contents of rats and so had effects on hemoglobin concentrations and hematocrit (Ohta *et al.*, 1995). Nevertheless values of hemoglobin concentration and hematocrit in the inulin group still were lower than the normal range given by the investigating laboratory for their analysis methods.

Immune- and inflammation modulatory effects of prebiotic substances – especially for inulin and oligofructose – have been reported repeatedly (e.g. Schley and Field, 2002). For example Field *et al.* (1999) reported changes in composition and function of gut associated lymphoid tissue in dogs fed a diet rich in fermentable fiber. Also an increase in expression of IgA and alterations of cytokine expression patterns was reported in murine peyer's patch cells after feeding of fructooligosaccharide (Hosono *et al.*, 2002). In the present study leukocyte counts were decreased in lactulose treated calves. A similar effect has been found when mannanoligosaccharides from yeast cell wall preparations were fed to dogs (Middelbos *et al.*, 2007). There prebiotic supplementation also led to a decrease in monocyte numbers as was found in inulin as well as in lactulose fed calves. Middelbos *et al.* (2007) suggest that a lowering of infectious load in the intestine can be achieved by mannan moieties. This in turn may decrease the necessity of activated immune cells in the vicinity, which may also be reflected in peripheral blood. A similar decrease in pathogen load in the intestine is also attributed to prebiotics such as inulin or lactulose (Bovee-Oudenhoven *et al.*, 1997; Flickinger *et al.*, 2003). Strengthening these findings, the expression of IL2RA – an activation marker on the surface of lymphocytes – tended to be lowered in the colon of lactulose treated calves compared to the control. This reduction also hints to a decreased pathogenic load in the intestine leading to lowered lymphocyte activation. This finding is also consistent with Fleige *et al.* (2009) who found a significantly lowered expression of IL2RA in the mesenteric lymph nodes of lactulose treated calves.

A similar pattern as for leukocyte counts and IL2RA expression could be found for TNF expression in jejunum. Lactulose significantly reduced its expression confirming again a decreased activation of an inflammatory or immune response in the intestine. A similar effect on TNF has been found before in a rat colitis model after treatment with lactulose (Camuesco *et al.*, 2005). In contrast pro-inflammatory IL8 was significantly enhanced in mesenteric lymph nodes of animals in the lactulose group. Fleige *et al.* (2009) also reported increases of IL8 expression in spleen and mesenteric lymph nodes of calves after feeding of lactulose, but these results were not statistically significant. IL8 expression has been

shown to be increased by lipoteichoic acid (Standiford *et al.*, 1993) of gram positive pathogens such as bifidobacteria which are reported to be promoted by lactulose (Camuesco *et al.*, 2005). Antigens of these bacteria may have been transported to the mesenteric lymph nodes by antigen presenting cells to elicit such a signal. But in fact no increase of IL8 mRNA was found in gut tissue. Additionally inulin also has been reported to enhance bifidobacteria numbers (Campbell *et al.*, 1997), but did not show a similar effect on IL8. Further, strains of bifidobacteria and lactobacilli have been shown not to induce IL8 expression in an epithelial cell line (Lammers *et al.*, 2002). And since no induction of IL8 was found in gut tissues, where these bacteria should show a first effect, it has to be considered whether other factors possibly independent of gut flora may be responsible for the significant increase of IL8 expression in mesenteric lymph nodes of lactulose fed calves.

Anti-inflammatory IL10 showed an increased expression in the ileum of inulin treated calves especially when compared to lactulose treated animals. The ileum wall is interstratified with peyer's patches, parts of the gut associated lymphoid tissue. Cells from theses patches have been shown to release anti-inflammatory cytokines such as IL10 after stimulation with probiotics, prebiotics or fermentation products thereof (Hosono *et al.*, 2003; Roller *et al.*, 2004; Säemann *et al.*, 2000). Interestingly lactulose had no or – compared to inulin – only minimally decreasing effects on IL10 production in the present study. This is consistent with the findings of Fleige *et al.* (2009), who also found a slight down-regulation of IL10 expression in ileum of lactulose treated calves. Yet the same author also found an increase in IL10 expression in the jejunum and colon of these animals (Fleige *et al.*, 2007b).

PECAM1 is a cell surface molecule of leukocytes and endothelial cells and is crucial for the transmigration of leukocytes through the capillary wall into inflamed tissue (Wakelin *et al.*, 1996). Regarding this an up-regulation of its expression as seen in both prebiotic feeding groups would have to be considered a pro-inflammatory event. On the other hand it has been shown that surface expression of PECAM1 is down-regulated on endothelial cells in typical pro-inflammatory situations such as stimulation with TNF or IFNG without effects on leukocyte transmigration. PECAM1 as a cell junction molecule seems to play a role in permeability of endothelial walls during inflammatory processes (Shaw *et al.*, 2001). But this molecule also plays a role in angiogenesis (DeLisser *et al.*, 1997), and therefore an up-regulation could also mean an increase in perfusion of the surrounding tissue. Probiotic *Lactobacillus rhamnosus* has been found to enhance gastric ulcer healing by stimulation of angiogenesis (Lam *et al.*, 2007). Maybe there an increase in PECAM1 expression may also play a role suggesting that its increase in the present study may be stimulated by beneficial lactic acid bacteria propagated by the prebiotic substances.

Expression of the proliferation marker MKI67 in ileum was increased in the inulin group. Interestingly morphologic investigations during the same study rather showed a slight decrease of villus length and number of proliferative cells in the same group, but an increase of both in the lactulose group (Masanetz *et al.*, 2010) which is also not mirrored in the mRNA expression. Maybe main MKI67 expression was not located in the epithelial layer, which was investigated by microscopy, but in other parts of the gut wall and was such veiling the results found only in the mucosa. Nevertheless both enhancement (Brunsgaard and Eggum, 1995) and decline (Femia *et al.*, 2002) of gut epithelial proliferation have been described after treatment with different prebiotic substances in animal models. Simultaneously a trend for an increased expression of BAX mRNA has been found in the ileum in both prebiotic groups. It has been reported before that butyrate – a fermentation product of prebiotic substances – is able to increase apoptosis via up-regulation of BAX expression (Mandal *et al.*, 2001). This is fitting to the proposed anti-carcinogenic effects described for inulin (Femia *et al.*, 2002). Fleige *et al.* (2007b) also found signs for pro-inflammatory processes in the intestine of calves fed lactulose, but there BAX expression was not significantly altered.

In conclusion both prebiotic substances used in the present study showed effects on immune regulation and inflammation both systemic and locally in the gut of calves. Inulin and lactulose decreased immune activation and increased anti- or lowered pro-inflammatory signals respectively. Presumably these effects were generated by a

decline of pathogen load in the intestine commonly attributed to prebiotic treatment. Effects of both substances on proliferation and apoptosis are rather diverse and remain to be studied in detail. Also positive effects on hemoglobin and hematocrit have only been shown for inulin. These presumed effects on iron absorption are highly interesting and remain to be studied in future experiments especially with a look on other mineral absorption capabilities. On the whole a beneficial effect of prebiotic feeding on veal calf health can be expected for both inulin or lactulose regarding the results of the present study.

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

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

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

target	orientation	sequence	size [bp]	accession	profile
		reference genes			
ACTB	for	AACTCCATCATGAAGTGTGACG	234	NM_173979	60/68
	rev	GATCCACATCTGCTGGAAGG			
GAPDH	for	GTCTTCACTACCATGGAGAAGG	197	NM_001034034	60/68
	rev	TCATGGATGACCTTGGCCAG			
UBIQ	for	AGATCCAGGATAAGGAAGGCAT	198	NM_174133	60/68
	rev	GCTCCACCTCCAGGGTGAT			
VPS4A	for	CAAAGCCAAGGAGAGCATTC	222	NM_001046615	61/68
	rev	ATGTTGGGCTTCTCCATCAC			
GAK	for	TCTGGGAAGTGGCAGAGAGT	294	NM_001046084	61/68
	rev	CGGCACGTCTGGTAGAAGAT			
RAB21	for	CGGAAAATGTTGGGAAACG	229	XM_001249323	61/68
	rev	CATTGCCTTTTGCCCTCTC			
PMPCA	for	CATCCCAGAATAAGTTTGGACAG	236	NM_001076964	61/68
	rev	AGAATCAGCAGACACAGCATACA			
		genes of interest			
IL1B	for	TTCTCTCCAGCCAACCTTCATT	198	NM_174093	61/68/80
	rev	ATCTGCAGCTGGATGTTTCCAT			
TNF	for	CCACGTTGTAGCCGACATC	155	NM_173966	61/68/80
	rev	ACCACCAGCTGGTTGTCTTC			
IL8	for	ATGACTTCCAAGCTGGCTGTTG	149	NM_173925	61/68/80
	rev	TTGATAAATTTGGGGTGGAAAG			
TGFB1	for	ACGTCACTGGAGTTGTGCGG	166	NM_001166068	61/68/80
	rev	TTCATGCCGTGAATGGTGGCG			
IL10	for	CCTGGAAGAGGTGATGCCAC	118	NM_174088.1	61/68/80
	rev	GTTTTCGCAGGGCAGAAAGCG			
IFNG	for	CTTGAATGGCAGCTCTGAGAAAC	173	NM_174086	61/68/80
	rev	GGCCTCGAAAGAGATTCTGAC			
FCAR	for	GACAAACCCTTTCTCTCCACC	180	NM_001012685	61/68/80
	rev	ACAGGACCCAGAGTGAAGTC			
IL2RA	for	ATGGAGCCAAGCTTGCTGATGT	171	NM_174358	61/68
	rev	TCTGCGGAAGCCTGTCTTGCA			
CD69	for	GTCATTGATTCTAAAGAGGACATGA	137	NM_174014	60/68
	rev	AGGTTGAACCAGTTGTTAAATTCT			
CD4	for	GATCGAGGTCTTGCCTTCAG	237	Multi (consensus)	61/68/80
	rev	GATCTGAGACATCCGTTCTGC			
CD8b	for	ACTGTGTATGGCAAGGAGGTG	127	NM_001105344	61/68/80
	rev	GGGTATCCCAATGATCATGCAG			
PECAM1	for	AAGGGAGGCATGACTGTGTC	187	NM_174571	61/68/80
	rev	TAATCACCTCGGACCTGGAG			
EGFR	for	AACTGTGAGGTGGTCCTTGG	173	XM_592211	61/68/80
	rev	AAAGCACATTTCCTCGGATG			
BCL2L1	for	GGCATTCAGCGACCTGAC	203	NM_001077486	61/68/80
	rev	CCATCCAAGTTGCGATCC			
BAX	for	TCTGACGGCAACTTCAACTG	203	NM_173894	61/68/80
	rev	GGTGTCCCAAAGTAGGAGAGG			
CASP3	for	GCAACGTTTCTAAAGAAGACCATAG	64	NM_001077840	60/68
	rev	CCATGGCTTAGAAGCACACAAATAA			
MKI67	for	TGGCGAAGATGTGTTTCCT	130	XM_590872	60/68

	rev	CGTGCTCCTTGGTGTTTTC

Table 3 *Results of hematological examination of blood samples taken ten weeks after beginning or during slaughtering. All values are presented as mean with r.e.m. (n=14). P values are results of ANOVA analysis, superscripts show group differences (Tukey-Kramer test).*

ten weeks after beginning	control	inulin	lactulose	r.e.m.	P values
erythrocytes, 10^6 µL^{-1}	8.8	9.2	8.9	1.07	n.s.
thrombocytes, 10^3 µL^{-1}	753.1^a	553.2^b	661.4^b	181.79	0.008
leukocytes, 10^3 µL^{-1}	8.9^a	9.6^a	6.5^b	2.40	0.005
basophiloc granulocytes, %[1]	0.9	0.6	0.6	0.46	n.s.
eosinophilic granulocytes, %[1]	2.4	2.1	2.4	1.88	n.s.
segmented granulocytes, %[1]	28.2	31.3	31.6	9.13	n.s.
lymphocytes, %[1]	61.4	61.4	60.1	10.75	n.s.
monocytes, %[1]	6.4	4.1	4.8	2.70	0.073
hemoglobin, g/dL	7.1	7.5	6.8	1.25	n.s.
hematocrit, %	17.1	18.6	16.4	3.04	n.s.
at slaughtering	**control**	**inulin**	**lactulose**	**r.e.m.**	**P values**
erythrocytes, 10^6 µL^{-1}	11.2	12.2	11.8	1.22	n.s.
thrombocytes, 10^3 µL^{-1}	455.3	577.4	654.4	302.65	n.s.
leukocytes, 10^3 µL^{-1}	5.3	6.2	6.9	2.45	n.s.
basophiloc granulocytes, %[1]	0.6	0.6	0.8	0.55	n.s.
eosinophilic granulocytes, %[1]	3.5	3.8	3.5	2.67	n.s.
segmented granulocytes, %[1]	41.2	42.9	47.6	13.35	n.s.
lymphocytes, %[1]	49.7	47.9	42.6	14.30	n.s.
monocytes, %[1]	4.6	4.5	4.5	2.21	n.s.
hemoglobin, g/dL	7.7^a	8.7^b	8.1^a	0.94	0.003
hematocrit, %	21.8^a	24.2^b	21.6^a	2.80	0.035

[1]of total leukocytes

Table 4 *Changes in relative mRNA expression levels found in the present study. All values are presented as geometric means with r.e.m. (n=14) of relative expression values with respect to the control levels. P values are results of ANOVA analysis, superscripts show group differences (Tukey-Kramer test).*

tissue	gene	control	inulin	lactulose	r.e.m.	P values
jejunum	TNF	1.0^{ab}	1.1^{b}	0.7^{a}	0.52	0.018
ileum	IL10	1.0^{ab}	1.5^{b}	0.8^{a}	0.91	0.048
ileum	BAX	1.0	1.3	1.3	0.45	0.096
ileum	MKI67	1.0^{ab}	2.0^{b}	0.8^{a}	1.13	0.009
colon	PECAM1	1.0^{a}	1.3^{b}	1.3^{b}	0.42	0.023
colon	IL2RA	1.0	0.9	0.7	0.98	0.058
mesLN	IL8	1.0^{a}	1.0^{a}	1.4^{b}	0.57	0.029

Appendix IV

Markus W., Hruz T., Docquier M., Pfaffl M.W., Masanetz S., Borghi L., Verbrugghe P., Kalaydjieva L., Bleuler S., Laule O., Descombes P., Gruissem W., Zimmermann P. (2010):

RefGenes: an online tool to identify reliable and condition specific reference genes for quantitative RT-PCR.

Nucleic Acids Research: Submitted

Expression stability is context-dependent and determines the choice of reference genes for RT-qPCR data normalization

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Abstract

Background: RT-qPCR is a sensitive and increasingly used method for gene expression quantification. To normalize RT-qPCR measurements between samples, most laboratories use endogenous reference genes as internal controls. There is increasing evidence, however, that the expression of commonly used reference genes can vary significantly in certain contexts. Using the Genevestigator database of normalized and well-annotated Affymetrix microarray experiments, we describe the expression stability characteristics of the transciptomes of several organisms. The results show that a) no genes are universally stable, b) most commonly used reference genes yield very high transcript abundances as compared to the entire transcriptome, and c) for each biological context a subset of stable genes exists that has smaller variance than commonly used reference genes or genes that were selected for their stability across all conditions. We therefore propose the normalization of RT-qPCR data using reference genes that are specifically chosen for the conditions under study. RefGenes is a community tool developed for that purpose. Validation RT-qPCR experiments across several organisms showed that the candidates proposed by RefGenes generally outperformed commonly used reference genes. RefGenes is publicly available at www.genevestigator.com.

Background

Rationale for using reference genes

Reference genes, sometimes also called "housekeeping genes", frequently serve as internal controls in transcript quantification assays such as RT-qPCR. The need for internal controls in such assays arises from sample to sample biases related to variability in total RNA content, RNA stability, enzymatic efficiencies, or sample loading variation. To correct for this, the expression levels measured are frequently normalized to internal control genes. Ideally, such genes are expected to be invariable in their expression and therefore correlate strongly with the total amounts of mRNA present in each sample. Commonly used reference genes, such as beta-actin (ACTB), ubiquitin (UBQ), the 18S ribosome small subunit (18S), beta-glucuronidase (GUS), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), have a strong tradition and historical track record. In fact, many manufacturers provide "housekeeping gene panels" containing a dozen such genes thought to be generally stable based on their biological function. In many laboratories, they are used as "general purpose" reference genes for a wide variety of experimental conditions.

Problems associated with reference genes

Despite their wide-spread use, the suitability of reference genes for any type of experiment is not given *a priori*. In fact, two types of problems can occur: 1) their expression can vary considerably depending on the experimental condition being tested, and 2) the majority of these genes is very strongly expressed, often resulting in a discrepancy in transcript abundance of several orders of magnitude relative to the target gene transcripts being quantified. Both sources of error can cause significant biases that can ultimately lead to wrong data interpretation, especially in those cases where a single gene is used for normalization. For example, [1–5] have described various problems associated with commonly used reference genes.

Current approaches for improved data normalization

Although limitations are universally recognized, still many laboratories use reference genes without appropriate validation [6, 7]. In an effort to improve the quality and normalization of RT-qPCR data, several approaches have been proposed.

A first approach consists of validating reference genes using data obtained from RT-qPCR data. Frequently, several genes are evaluated in parallel and the most stable are selected for further experimentation. So far, most studies have focused on validating a subset of commonly used reference genes for specific contexts such as tissue types. Overall, it appears that no reference gene was generally suitable for any type of context, and that the best candidates differ between different tissues. In some cases, even opposite results were found for different tissues. For example, Meller et al. [3] analyzed seven commonly used reference genes for their expression level stability in placenta and reported that TBP and SHDA exhibited highest stability. In contrast, of the 10 commonly used reference genes tested by Zhang and colleagues in human neutrophils [5], TBP appeared to be the least stable. A list of similar studies in which validations were performed in a variety of organisms and tissues is available in Web supplement 1. Although these studies have their merits, they try to identify the best candidates from a small and *a priori* set of genes, assuming that at least one or a few of them are suitable for the experimental context under study.

A second approach is to normalize against multiple reference genes and to use appropriate statistical models to improve the selection of genes with minimal variance [8–14]. Most current software packages for RT-qPCR data analysis have incorporated one or the other of these methods. Three of the most popular algorithms are GeNorm [13], Normfinder [8] and Bestkeeper [15].

A more recent, data-driven method consists of using quantile normalization rather than reference genes, but this approach is designed for high-throughput RT-qPCR experiments involving many genes. For studies involving one or a few genes, data normalization using internal control genes remains the method of choice, provided a proper choice of reference genes and normalization algorithms [16, 17].

A fourth and quite successful approach has been to search for reference genes from a genome-wide background using microarray data. In most cases, large sets of microarray data were compiled for a specific or for a subset of conditions, and stable genes identified within these datasets were validated and recommended for future use. Validation experiments generally showed that these genes performed better than commonly used reference genes. For example,

Czechowski and colleagues [18] selected stably expressed genes for a variety of experimental series for Arabidopsis. Partial overlap was found between some of these conditions, but overall each series had its specific set of most stably expressed genes. Saviozzi et al. [19] performed a meta-analysis of lung cancer transcription profiles and validated several new reference genes for this particular context. Other similar studies were done e.g. for T-helper cells [20], adipose tissues [21], peripheral blood [22], various human samples and cell lines [23], breast tumor tissues [24], breast cancer [25], human myocardium [26], mouse (universal) [27], and human (universal) [28].

Conclusions from published data

From the experimental evidence accumulated and published so far, we conclude that there are probably no genes that have a sufficient overall expression stability to be suitable for any type of assay. As previously suggested, reference genes should be selected according to the nature of the study [6, 7], for example according to the tissue type or stage of development, and should ideally not be sensitive to perturbations such as external stimuli, diseases, or even to genetic modifications. Moreover, reference genes are preferably selected from the complete genome rather than from a handful of commonly used reference genes.

Hypotheses

In this study, we have examined how to find the best possible candidate reference genes for specific experimental contexts, starting from a genome-wide set of genes. To do so, we defined an "ideal reference gene" as a gene which 1) has the most stable transcript abundance within the biological context of a specific experiment, and 2) has an abundance of transcripts similar to that of the target genes under investigation. The hypotheses that we tested were the following:

1. No genes are generally stably expressed; all genes are regulated to a certain extent (non-generality clause)

2. For each biological context there exists a subset of genes with smaller expression variance in this context than genes that are stably expressed across many conditions (context-specificity clause)

3. Genes that are stably expressed in a given biological context are likely to be stably expressed in similar contexts (context- relatedness clause)

4. Genes that are stably expressed in a given tissue of an organism are likely to be stably expressed in the same tissue from closely related species (orthology clause)

In this paper, we tested and substantiated these hypotheses by using data from more than 40,000 quality controlled and manually annotated microarrays from a wide variety of experimental contexts and from several organisms. We studied the properties of the expression level of genes across various microarray types. Finally, to validate our approach, we identified novel reference genes, examined their individual properties, and compared their performance to commonly used reference genes using RT-qPCR assays. We also present an online tool which helps to identify genes that show high expression stability in a chosen set of conditions. Researchers can thereby identify, from all genes represented on the microarrays, those which are most stably expressed across conditions that are similar to that of their own experiments, providing them with an objective choice of candidate reference genes.

Results

Datasets used in this study

The Genevestigator database contains a large set of systematically annotated and quality controlled microarray data from several organisms [29]. Owing to the high reproducibility of the Affymetrix system, its streamlined labeling and hybridization protocols, the normalization methods used, as well as our quality control measures, expression data from different laboratories show a high degree of homogeneity. The database therefore offers a unique opportunity to search for genes that have particular expression characteristics across experiments, for example reference genes that have minimal variance across a chosen set of conditions.

Validating our hypotheses

Hypothesis 1 (non-generality clause)

Public experimental evidence accumulated and published so far seems to suggest that there are no

genes whose expression is universally stable across any type of condition. To verify this hypothesis, we measured the standard deviation of gene expression across large sets of Affymetrix arrays from various array types and organisms and covering a broad variety of conditions. The results show that for all organisms tested, the ranges of standard deviation of gene expression across the complete available datasets were approximately 15-fold, with values mostly varying between 0.5 and 5 (Figure 1; see also Web supplement 2). Commonly used reference genes were generally located within the range of SD between 0.5 and 1.0. However, in the human dataset shown in Figure 1, more than 8000 other probe sets were also located within this range of SD. It is unlikely that the expression of one fifth of the transcriptome is sufficiently invariant so that any of them could be used for normalization. Furthermore, no genes were found to have a standard deviation distinctly lower than the remaining genes. Genes with a high average expression level showed slightly lower variance of expression across these datasets. This effect could be due to the normalization method used in this study (MAS5) or to saturation effects. Nevertheless, it is interesting to note that most reference gene panels tend to choose very highly expressed genes. In some of the plots, a partly distinct cloud of probe sets was formed in the very high range of expression and low range of standard deviation. This cloud is enriched in cross-hybridizing probe sets, mainly probe sets hybridizing transcripts from the same family of genes. The vast majority of them represent genes encoding ribosomal proteins, while from the remaining genes from this cloud several commonly used reference genes were identified, such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ACTB (beta-actin), UBB (ubiquitin B), B2M (beta-2-microglobulin), PPIA (peptidylprolyl isomerase A (cyclophilin A)), EIF1 (eukaryotic translation initiation factor 1), TUBA1B (tubulin, alpha 1b), HSP90AA1 (heat shock protein 90kDa alpha (cytosolic), class A member 1), UBC (ubiquitin C), H3F3A (H3 histone, family 3A) and EEF1G (Eukaryotic translation elongation factor 1 gamma). Similar observations were obtained by analyzing data from various array types and organisms, including human, mouse and Arabidopsis (see Web supplement 2). A further evidence supporting this hypothesis is that from the top 50 most stable transcripts identified from Figure 1 (see Web supplement 4), all of them were found to have a highly variable expression in at least five conditions or tissues available in Genevestigator. These genes would clearly not be suitable to normalize data obtained from these particular experimental conditions, even if their overall expression stability is high.

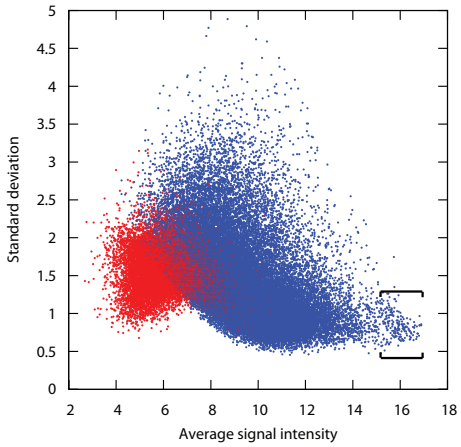

Figure 1. Standard deviation versus average transcript abundance of approximately 47,000 probe sets across 5014 Affymetrix Human133 2.0 arrays. Spots in blue show probe sets with Present calls in at least ten percent of arrays, while spots in red are those with Absent calls in more than ninety percent of arrays. The probe sets contained between the square brackets are highly enriched in ribosomal protein genes, but include many of the commonly used reference genes (e.g. GAPDH, ACTB, B2M, PPIA, EIF1, ACTG1, UBC, EEF1G, TUBA1B, EEF1A1, TPT1).

Hypothesis 2 (Context-specificity clause) Our second hypothesis is that for each biological context, a distinct set of genes exists with lower variance within this context than genes selected for their stability over a variety of different contexts. To verify this hypothesis, we created, from a compendium of 3051 mouse arrays (Mouse430 2.0) from Genevestigator, selections of arrays representing various tissue types (muscle, liver, lung, fibroblast, Central Nervous System). As a control, we created a selection of all 3051 arrays covering a wide variety of contexts. We chose to work with the mouse dataset because it contained several tissue types with high data coverage. For each of these array selections, we calculated the standard deviation (SD) for each probe set available on the array and ranked them from lowest to highest SD. Figure 2A shows the results for 20 commonly used reference genes across all arrays (a), and across tissue-specific

subsets of arrays (b). In Figure 2B, for each tissue type we identified the top-20 genes with lowest SD and ranked them by increasing SD (d), and as a control, we show their respective ranked SD across all arrays (c). Two observations can be made:
1) Genes selected for their stability within a chosen tissue type had lower SD of expression than commonly used reference genes, both within these tissue types (up to 4-fold lower) and also as measured across all arrays (up to 1.5 fold lower). 2) For each tissue, the range of SD of the top 20 most stable transcripts was within 1.5 fold difference between the most stable and least stable gene (see also Web supplement 6). In contrast, the SD of the 20 commonly used reference genes varied more than 5-fold, irrespective of the tissue type, indicating that for each tissue type several of these genes would be unsuitable for data normalization. None of the 20 commonly used reference genes was systematically ranked within the top 5 genes across every tissue type, and some even had highly variable ranks. For example, TFRC had rank 1 in spinal cord and rank 20 in liver (see Web supplement 5).

To substantiate these findings, we carried out two independent RT-qPCR experiments with tissues from mouse and Arabidopsis samples. For each experiment, we used the RefGenes tool from Genevestigator (see below) to find candidate reference genes for specific tissue types, and then tested these candidates against commonly used reference genes using GeNorm. The first experiment was carried out with mouse liver. The stability of four control reference genes (GAPDH, TUBB, ACTB, and HPRT) was compared to that of four novel reference genes (vps4a, srp72, mRpL16, and GAK) identified as being highly stable across a set of 197 Affymetrix arrays profiling mouse liver samples from 7 distinct public experiments available in Genevestigator. For each gene, measurements were done in triplicate for 16 liver samples, and all reactions were run simultaneously. From these eight genes, GeNorm iteratively removed the least stable ones in the following order: TUBB, GAPDH, HPRT, ACTB. The three most stable genes in mouse liver were: srp72, mRpL16 and GAK, whereas in almost every iteration GAK appeared to be the most stable gene (see Web supplement 3). This experiment proved that liver-specific stable genes, as identified from Affymetrix microarray data from liver samples, outperformed commonly used reference genes for the normalization of RT-qPCR data from liver.

The second experiment consisted of identifying genes that are stable in seedlings, leaves and shoot apex of the model plant Arabidopsis, and to compare their expression with that of reference genes commonly used in this model plant using RT-qPCR. For each tissue type, 16, 16, and 10 samples were used, respectively. The results are provided in Table 1. For seedlings and shoot apex, all candidates proposed by RefGenes showed higher stability in this experiment than the reference genes GAPDH, ACTB and UBQ10. In leaves, GAPDH appeared to be most stable, with RefGenes candidates in the second and third ranks. These results confirm those obtained from mouse liver tissue, i.e. that the tissue-specific selection of reference genes using microarray data allows to identify novel genes having higher expression stability and a more suitable expression range than most commonly used reference genes. In fact, for both organisms and across all genes tested, the Cp values (i.e. the number of PCR cycles that elapse before a given threshold concentration of PCR product is reached) from the novel RefGenes candidates were higher than those of commonly used reference genes.

Hypothesis 3 (Context-relatedness clause)
Our third hypothesis is that related tissue types have similar sets of genes that are most stable within these tissues. To verify this hypothesis, we selected 14 different tissue types for which at least 100 arrays from minimally 3 independent experiments were available, and compared the overlap of the top 20 and top 50 genes that were most stable in each of them (Figure 3). As a control, we selected the top 20 and top 50 genes, respectively, that were most stable across all arrays. On average, the SD of expression within each subset of arrays increased 30% between probe sets of rank 1 and rank 20, and 43%, 54% and 67% between rank 1 and rank 50, 100 and 200, respectively (see Web supplement 6). The results show that in the top 20 comparisons, very few genes overlapped between any of the tissue types. In the top-50 comparisons, biologically related tissues such as spinal cord and central nervous system, splenocytes and leucocytes, or leucocytes and fibroblasts, had a higher overlap of the most stable genes between these tissues than between unrelated tissue types. It was also observed that only few genes that were selected to be most stable across all arrays (category ALL in Figure 3) were also found in the top 20 or top 50 genes from each tissue type. The highest

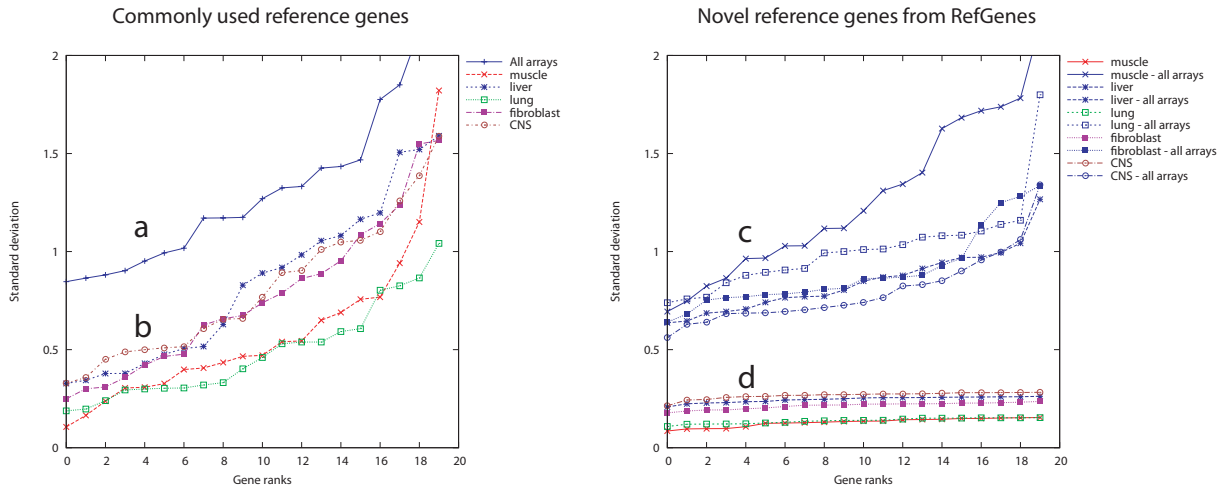

Figure 2. Standard deviation (SD) of gene expression of commonly used reference genes (A) across all arrays (a) or across subsets of tissue-specific arrays (b) from the Affymetrix Mouse430 2.0 array. In (B), for each subset of tissue-specific arrays the most stable genes were identified using Genevestigator RefGenes. Their respective expression SD across all arrays (c) or across subsets of tissue-specific arrays (d) is shown. The control reference genes used in this study and shown in plot A were: HSP90AB1, TFRC, B2M, NONO, GUSB, UBC, ACTB, H2AFZ, POLR2A, TUBB4, HIST2H2AA1, RPL22, GAPDH, YWHAZ, CANX, CYC1, SDHA, EIF4A2, ATP5B, and EEF1E1.

such overlap was found in macrophages, spinal cord, and liver.

The feasibility of selecting reference genes using related tissues is also illustrated in the following example. For human B-lymphocytes, only 4 arrays were available in the human 47k dataset. We therefore chose to work with an extended set of tissues that were the most closely related to B-lymphocytes as identified by clustering the Genevestigator anatomical profiles of 10 randomly chosen sets of 400 genes. 46 arrays covering three closely related tissue types (B-lymphocytes, 4 arrays; lymphoblast cells, 24 arrays; lymphocytes, 18 arrays) were selected. Six novel candidate reference genes proposed by RefGenes were selected for this study and were compared to five commonly used reference genes (SDHA, GAPDH, YWHAZ, B2M, RPL13a). The RT-qPCR validation experiment was carried out on lymphoblastoid cell lines (LCLs) of 15 subjects. The results of the top 8 genes as selected by GeNorm are shown in Table 1. Two of the candidate genes obtained from RefGenes performed best and yielded significantly lower M values in GeNorm than the other reference genes. The remaining RefGenes candidates were similarly or less stably expressed than the control reference genes. Although in the microarray data (comprising several tissue types) all candidates proposed by RefGenes were more stable than commonly used reference genes, in this particular experiment based on LCLs only, the ranking of variances was different. This illustrates that expanding the search to related tissues has the potential to yield significantly better candidates, but it may be necessary to test a larger number of candidates, as some of them may be of similar or lower quality than commonly used reference genes. It must be noted, however, that not only the variance, but also the expression intensity range should be considered in choosing a reference gene. In fact, the commonly used reference genes tested had lower Cp values (reflecting very high expression levels), and therefore the novel RefGenes candidates could be preferred if their Cp values are closer to those of a specific target gene and their variances are similar to alternative reference genes.

		Rank of the average expression stability values of remaining reference genes								Mean values for		
	Samples	1	2	3	4	5	6	7	8	Top 3 genes	RefGenes candidates	Common ref. genes
SPECIFIC TISSUES												
Mouse liver	16	GAK	SRP72	mRpL16	VPS4A	ACTB	HPRT	GAPDH	TUBB			
GeNorm (Avg M)		*0.15*	*0.15*	*0.17*	*0.19*	*0.21*	*0.24*	*0.27*	*0.30*	*0.16*	*0.17*	*0.26*
Mean Ct		25.02	24.68	26.56	26.91	20.47	25.09	19.50	24.41	25.42	25.79	22.37
Arabidopsis seedling	16	At3g24160	At1g13320	At3g27820	GADPH	ACTB	UBQ10					
GeNorm (Avg M)		*0.19*	*0.19*	*0.22*	*0.25*	*0.28*	*0.32*			*0.20*	*0.20*	*0.28*
Mean Ct		20.23	21.04	21.47	17.74	17.51	17.73			20.91	20.91	17.66
Arabidopsis leaf	16	GAPDH	At3g01150	At3g61710	ACTB	At1g32050	UBQ10					
GeNorm (Avg M)		*0.16*	*0.16*	*0.31*	*0.42*	*0.50*	*0.63*			*0.21*	*0.32*	*0.40*
Mean Ct		21.07	26.65	25.80	21.66	20.03	23.63			24.51	*24.16*	22.12
Arabidopsis apex	10	At2g17390	AT3G17920	At5g51880	ACTB	GADPH	UBQ10					
GeNorm (Avg M)		*0.11*	*0.11*	*0.15*	*0.20*	*0.22*	*0.49*			*0.12*	*0.12*	*0.30*
Mean Ct		18.89	23.14	22.22	17.91	17.92	21.86			21.42	21.42	19.23
RELATED TISSUES FROM SAME ORGANISM *(RefGenes search included B-lymphocytes and related tissues; qRT-PCR done on B-lymphocytes)*												
Human LCL + related	16	EIF4EBP2	INTS4	SDHA	GAPD	YWHAZ	B2M	ZNF410	BUD13			
GeNorm (Avg M)		*0.08*	*0.08*	*0.12*	*0.14*	*0.16*	*0.17*	*0.18*	*0.20*	*0.09*	*0.14*	*0.15*
Mean Ct		23.64	26.10	23.52	16.65	21.10	15.79	24.38	26.07	*24.42*	*25.05*	*19.27*
SAME TISSUE FROM RELATED ORGANISM *(RefGenes identified genes from mouse liver data; orthologs were used for qRT-PCR in other species)*												
Cattle liver	42	VPS4A	GAK	ACTB	PMPCA	UBQ	GAPDH					
GeNorm (Avg M)		*0.25*	*0.25*	*0.27*	*0.29*	*0.32*	*0.35*			*0.26*	*0.26*	*0.31*
Mean Ct		16.20	17.05	11.99	17.45	11.52	13.32			15.08	16.90	12.28
Pig liver	48	Histone H3	UBQ	VPS4A	GAK	GAPDH	PMPCA					
GeNorm (Avg M)		*0.29*	*0.29*	*0.30*	*0.32*	*0.34*	*0.36*			*0.29*	*0.33*	*0.31*
Mean Ct		13.10	9.68	17.41	16.98	16.87	18.08			13.40	17.49	13.22

Table 1. GeNorm and mean Cp values from the RT-qPCR validation experiments carried out on samples from Mouse, Arabidopsis, Human, Cattle, and Pig. Novel reference gene candidates (blue) identified with the RefGenes tool were compared to commonly used reference genes (yellow). The first section shows the results for tissues that were abundantly represented in Genevestigator and for which novel reference genes were proposed by RefGenes. In this case, the candidates proposed by RefGenes generally performed better according to GeNorm than commonly used reference genes. The second section shows RT-qPCR results for human lymphoblastoid cell lines (LCLs). Novel reference genes were identified from a a set of human LCLs and related tissues, because there were too few arrays available for this specific tissue type alone. The third section shows results in cattle and pig liver. Because at the time of writing this article no cattle and pig expression data were available in Genevestigator, novel reference gene candidates were identified with RefGenes from mouse liver samples and extrapolated to orthologs from cattle and pig. The GeNorm values indicated ("GeNorm (Avg M)") represent the average of the expression stability values M of the remaining genes after removal of the least stable one (see full results in Web supplement 3).

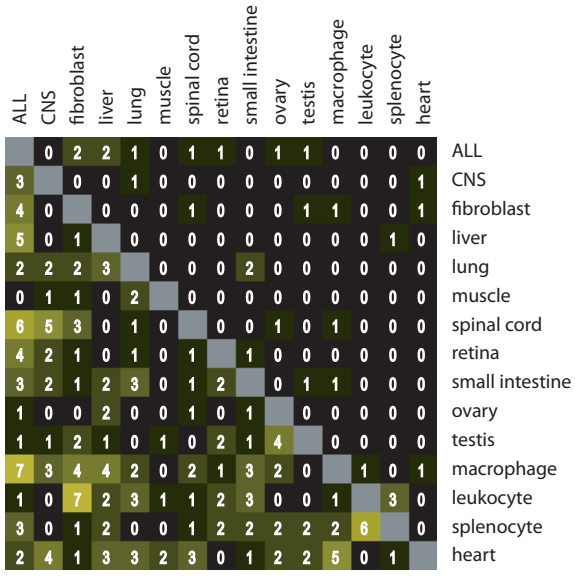

Figure 3. Overlap of the top 20 (top right section) and top 50 (lower left section) candidate reference genes identified by RefGenes in different tissues. A higher overlap can be observed between biologically related tissues. CNS: Central Nervous System.

Hypothesis 4 (orthology clause)

Our fourth hypothesis is that the stability of expression of gene orthologs remains similar across related species. Here, we cannot provide a general proof of principle, but an initial set of evidence to substantiate this hypothesis.

As a case study, we checked whether orthologs of genes that are highly stable in mouse liver could be used as alternative reference genes for qPCR experiments carried out on cattle liver and pig liver samples. In fact, although Genevestigator currently does not contain data from these species, we hy-

pothesized that the positive results obtained with mouse liver could be reproduced in other species by choosing the corresponding orthologs. Due to the incompleteness of available annotations for orthologs across these species, from the four genes that were previously validated in mouse, two (GAK and VPS4A) were found in cattle and pig. We identified a further gene (PMPCA) that was stable in mouse microarray data and was available as ortholog in cattle and pig. These three genes were compared to three commonly used reference genes (ACTB, GAPDH, and UBQ for cattle, and Histone H3, GAPDH and UBQ for pig) in a RT-qPCR experiment comprising 42 cattle liver samples and 48 pig liver samples. The application of both GeNorm and Normfinder to identify the most stable genes within the cattle dataset showed that the two best normalizers were GAK and VPS4A (Table 1; see also Web supplement 3). PMPCA performed similarly to commonly used reference genes. In pig, Histone H3, Ubiquitin and VPS4A performed best, followed by GAK, GAPDH and PMPCA. Overall, our results show that genes that are highly stable in mouse liver tend to have orthologs in other species that are also highly stable. This is particularly useful for those cases where the search for new reference genes is limited by the amount of microarray data available for a given species, but abundant data is available in related species.

The RefGenes tool

Our results suggest that for RT-qPCR it is best to identify specific reference genes for each experiment individually. To this end, we have developed RefGenes, a novel online tool from the Genevestigator platform. The main feature of RefGenes is to search for genes that exhibit minimal expression variance across a chosen set of arrays. Its graphical user interface is shown in Figure 4. RefGenes is very simple to use and requires only two main actions:
1) choosing a set of arrays
2) choosing the range of expression.

Choosing a set of arrays

The user can create selections of arrays according to organism and chosen sample properties, for example a set of human arrays from a particular tissue type. Currently, array selections can be done from sample annotations such as anatomical part, developmental stage, treatment, disease, genetic modification, or tumor type. Because the database is populated with a very large number of experiments, researchers can often identify subsets of arrays from a context similar to that from their own RT-qPCR experiment. Our recommendation is to select at least three independent studies comprising at least 60 arrays in total. If this cannot be reached within a specific context, it may be worth extending this context with closely related conditions. In the example shown in Figure 5, we selected 137 arrays hybridized with transcripts from CD4 T-Lymphocyte samples.

Choosing the range of expression

Theoretically, as long as data normalization is carried out in the linear range of amplification of both target and reference gene, it is not necessary for them to be in the same range of expression. However, this may not always be the case and may require the use of genes that are in a similar range of expression. In RefGenes, the user can define the upper and lower bounds of the search space such as to obtain candidate reference genes within these bounds. As an additional information, a bar below the graph indicates, for a given type of array, the typical ranges of low, medium, and high expression (where "Medium" indicates the interquartile range). We recommend to upload genes of interest as well as alternative reference genes for a comparison with new candidates that will be proposed by RefGenes. In the example shown in Figure 5, we uploaded the probe set identifiers for GAPDH, TUBB, PPIA, B2M, TBP, UBC, ACTB, RPL13A, as well as that of PIK3R1 as an example of a target gene to be measured by RT-qPCR in CD4 T-lymphocytes. We then defined the range of reference gene expression to be slightly above and below that of PIK3R1.

Searching for reference genes

The "Run" button allows to trigger the search algorithm based on the selections of arrays and genes. The Genevestigator engine searches for genes with the lowest variance within this selection of arrays and displays the top 25 probe sets. For each probe set, the mean and standard deviation are indicated. Mouse-over tooltips over each probe set provide additional information such as gene name and IDs for various gene models. In the present example, after launching the search by clicking on the "Run" button, RefGenes suggested 25 potential reference genes, of which the standard deviation of expression was between 0.22 and 0.31. As a comparison,

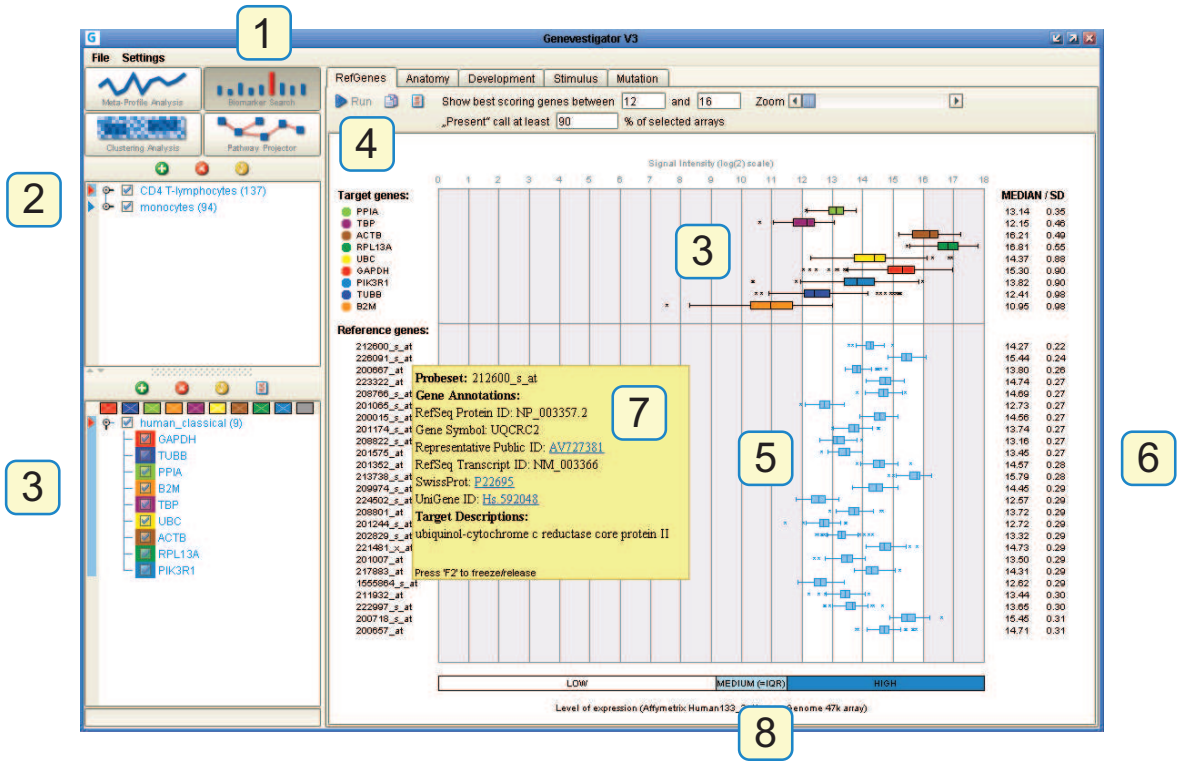

Figure 4. Graphical user interface of the RefGenes tool in Genevestigator. 1) The RefGenes tool belongs to the Biomarker Search toolset. 2) Panel for the selection of arrays associated with various experiments or conditions. 3) Panel for the selection of genes (target genes or commonly used reference genes for comparison). These genes are represented in the graph on the right with box and whiskers plots of signal intensity. In this example, the box and whiskers plots of expression in T-lymphocytes of 8 commonly used reference genes and a target gene (PIK3R1) are shown. 4) RefGenes toolbar, with fields to define the range of signal intensity within which new reference genes must be searched, and the "Run" button to start the search. 5) Box and whiskers plot of signal intensity of the new reference genes proposed by RefGenes. 6) The numerical values of the median and standard deviation of signal intensity are shown. 7) For each reference gene proposed by RefGenes, additional information is available in the mouse-over tooltip. 8) The typical range of low, medium, and high expression is shown for the array type chosen in (2). Medium is defined as the interquartile range (IQR).

the standard deviations of commonly used reference genes was between 0.35 and 0.98.

Validating potential reference genes

The candidate reference genes obtained can be partially validated by checking their expression across all microarrays available for that array type. This allows to verify whether there are particular conditions in which their expression varies unexpectedly. Figure 5 shows the results obtained for these reference gene candidates across various conditions depicted in Genevestigator (Anatomy, Development, Stimulus, and Mutation). In general, these genes appear to be very unresponsive to a wide variety of conditions. One of the genes is likely to be a less good candidate as it responds strongly to a subset of conditions. We also observed that most of the candidate genes had a slight response to various tumors and to oncolytic viruses.

Discussion

Our approach builds on previous studies showing that reference genes identified from microarray data often performed better in normalizing RT-qPCR experiments than commonly used reference genes. In contrast to previous studies, however, our approach combines three levels: 1) it searches for the most

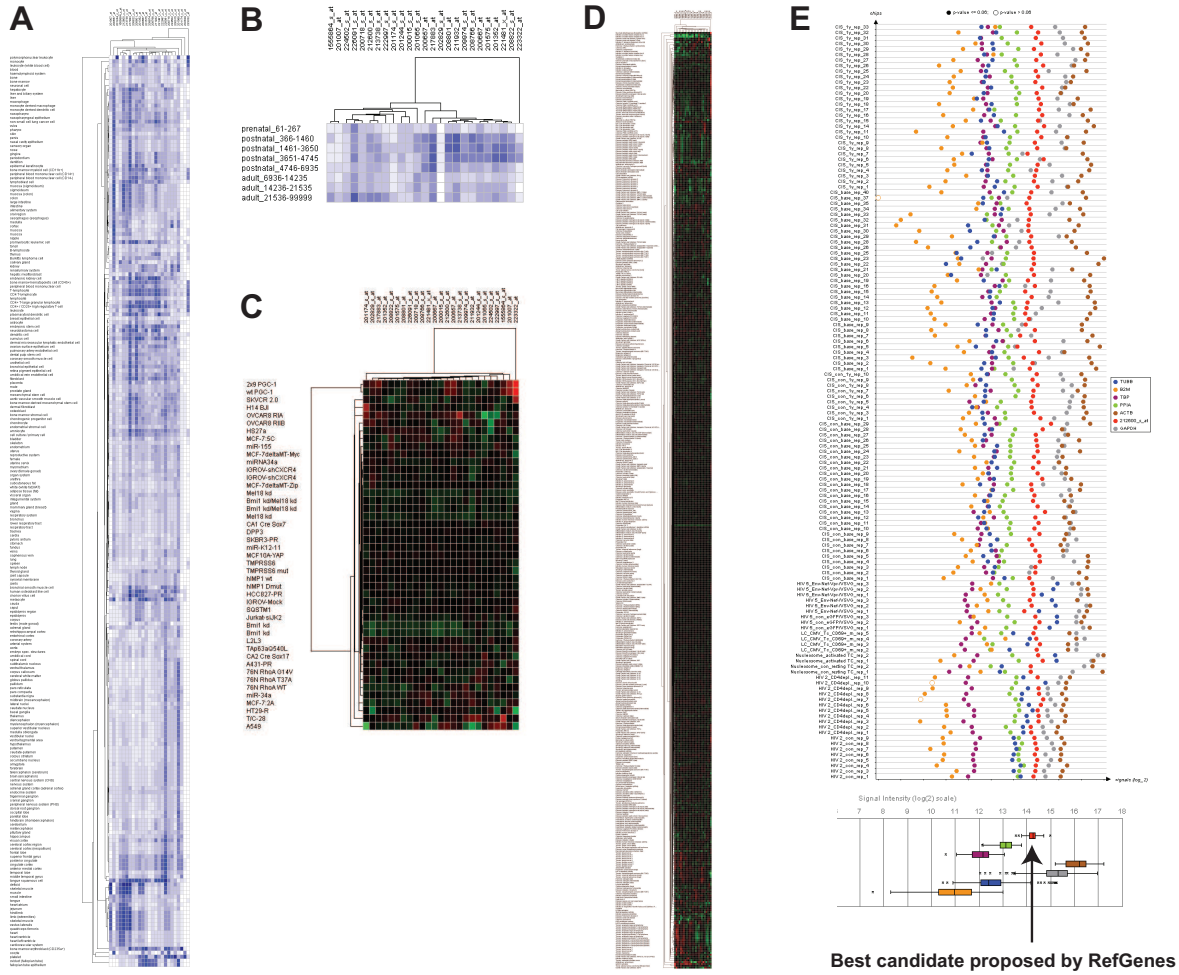

Figure 5. Validation of new reference genes identified for CD4 T-lymphocytes (137 arrays; 5 studies) A. Hierarchical clustering of the expression profiles of these 25 gene candidates across anatomical categories. B. Expression profiles across developmental stages. C. Response to various mutated cell lines. D. Response across a large set of diseases, chemical treatments, and tumors. E. Expression characteristics of several commonly used reference genes across the 137 arrays from CD4 T-lymphocyte samples. The box and whiskers plots from RefGenes for these genes is shown, and the best novel candidate reference gene identified by RefGene is shown in red and indicated with an arrow.

stable candidates from a genome-wide set of genes (rather than from a small set of commonly used reference genes), 2) it restricts the search to an expression range similar to that of own target genes, and 3) it allows users to flexibly choose, from a very large array compendium, context-specific sets of microarrays based on sample annotations. Additionally, based on the Genevestigator standardized data content, it allows users to cross-validate new candidates across a large set of experimental conditions prior to testing them in the laboratory. RefGenes therefore allows to select experimental conditions that are similar to that of a specific experiment and to obtain reliable and condition-specific candidates for the normalization of RT-qPCR or other types of transcript quantification data. Although Genevestigator currently contains more than 40,000 arrays, several experimental conditions may not yet be well populated (e.g. B-lymphocytes). In such cases, it is recommended to include additional arrays from related experimental conditions.

It is possible that the candidates proposed by Ref-

Genes are biased by the inherent nature of microarray data as compared to RT-qPCR data, or by data transformation procedures during normalization. In fact, one would expect variance to depend linearly on the mean based on original intensities (which are proportional to molecular concentration). Nevertheless, and despite differences in sensitivity between the two technologies, we did not observe major discrepancies that would question the use of microarray data to identify stably expressed genes to be used as references for RT-qPCR. In fact, the experiments described above, as well as previously published work (e.g. [18] demonstrate that the availability of quality controlled and normalized oligonucleotide microarray data (such as Affymetrix GeneChip arrays) allows to identify better reference gene candidates than commonly used reference genes. The use of different normalization methods or measures of variance is expected to influence the outcome of a search by RefGenes, but overall it is unlikely that genes that exhibit a high stability within a RT-qPCR experiment would not be identified by either of these methods at the microarray level. Here, we show a proof of principle of reference gene identification and validation using data normalized with MAS5 and RT-qPCR. Improvements in data processing in either of these technologies can be expected to yield even more robust results.

We therefore conclude that, for individual experimental conditions, it is worth searching for a number of new candidates and validating them against commonly used reference genes. The proposed general approach is illustrated in Figure 6: instead of starting with a handful of commonly used reference genes, we propose to start with a statistically selected, context-specific set of candidate genes identified by RefGenes, and then to validate them (optionally together with commonly used genes) within the experiment under study using algorithms such as GeNorm, Normfinder, or Bestkeeper. We also strongly recommend researchers to read the MIQE guidelines [17] as a guide to help carrying out and publishing their work.

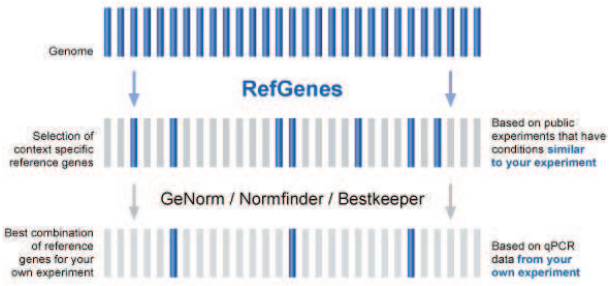

Figure 6. Proposed approach for the selection of suitable reference genes. Rather than starting from a subset of commonly used reference genes, we propose to start with an objective choice of candidate genes based on public microarray data obtained from similar experimental conditions. The second step remains identical, i.e. the validation of several candidates within the RT-qPCR experiment being carried out, and the selection of the most stable ones using algorithms such as GeNorm, Normfinder, or Bestkeeper.

Conclusions

We conclude that the identification of context-specific reference genes, combined with existing methods for normalization against multiple controls, is expected to significantly improve the quality and sensitivity of expression quantification experiments, facilitating the correct interpretation of RT-qPCR data. RefGenes is freely available at www.genevestigator.org.

Methods

Microarray data

Data from Genevestigator was normalized, quality controlled, and annotated manually as described previously [29]. In brief, Affymetrix expression array data used for this study was normalized using the MAS5 algorithm, with global scaling set to a target value of 1000. The quality of the arrays was assessed using various Bioconductor [30] packages, including AffyQCReport and SimpleAffy [31]. Sample descriptions were annotated using the Genevestigator application ontologies for anatomical parts, stage of development, and experimental perturbations. Novel reference gene candidates used for experimental validation were obtained from RefGenes. The search algorithm identifies, for a chosen set of microarrays, those probe sets for which the standard deviation of signal intensities across these arrays is lowest.

RT-qPCR for mouse liver

Livers were taken from WT and Reverb alpha mutant females fed with 2 different diets. RNA was extracted according to Fonjallaz's protocol [32]. cDNA was synthesized from 1 μg of total RNA using random hexamers and Supercript II reverse transcriptase (Invitrogen) following suppliers instructions. SYBR green assays were designed using the program Primer Express v 2.0 (Applied Biosystems) with default parameters such that they spanned exon bondaries when possible. Amplicon sequences were aligned against the mouse genome by BLAST to check for specificity. Oligonucleotides were obtained from Invitrogen. The efficiency of each design was tested with serial dilutions of cDNA. PCR reactions (10 μL volume) contained diluted cDNA, 2 x Power SYBR Green Master Mix (Applied Biosystems), 300 nM of forward and reverse primers. PCR were performed on a SDS 7900 HT instrument (Applied Biosystems) with the following parameters: 50 °C for two minutes, 95 °C for ten minutes, and 40 cycles at 95 °C for 15 seconds and 60 °C for one minute. Each reaction was performed in three replicates on a 384-wells plate. Raw Cp values obtained with SDS 2.2.2 software (Applied Biosystems) were analysed and the best house keeping genes selected according to the GeNorm method [13]. The forward (F) and reverse (R) primers used for this experiment were:

Mm GAK F CTGCCCACCAGGCATTTG
Mm GAK R CCATGTCACATACATATTCAATGTACCT
Mm MRPl46 F GGGAGCAGGCATTCCTACAG
Mm MRPl46 R GGTCCGGTCATTTTTTTTGTCA
Mm SRP72 F CACCCAGCAGACAGACAAACTG
Mm SRP72 R GCACTCATCGTAGCGTTCCA
Mm VPS4A F GACAACGTCAACCCTCCAGAAA
Mm VPS4A R TCTGTGGCTTTTGTCACCAGAT
Mm TUBB F GCAGTGCGGCAACCAGAT
Mm TUBB R AGTGGGATCAATGCCATGCT
Mm HPRT F GCTCGAGATGTCATGAAGGAGAT
Mm HPRT R AAAGAACTTATAGCCCCCCTTGA
Mm ACTB F CTAAGGCCAACCGTGAAAAGAT
Mm ACTB R CACAGCCTGGATGGCTACGT
Mm GAPDH F TCCATGACAACTTTGGCATTG
Mm GAPDH R CAGTCTTCTGGGTGGCAGTGA

RT-qPCR for human LCLs

Human lymphocytes were isolated from blood samples by Ficoll Lymphocyte Separation Medium (MP Biochemicals). Lymphoblastoid cell lines were obtained by transformation of the fresh lymphocytes with Epstein-Barr Virus and grown in advanced RPMI medium supplemented with 2% fetal bovine serum, 2 mM glutaMAX (L-Alanyl-L-Glutamine), 50 units/mL penicillin, 50 μg/mL streptomycin and 2% phytohemagglutinin, all from Invitrogen (Carlsbad, CA, USA). For extraction of total RNA, the transformed lymphoblastoid cell lines were harvested, lysed in RLT buffer (Qiagen, Valencia, CA, USA) and homogenized with a QIAshredder homogenizer (Qiagen). RNA purification was performed with the Qiagen RNeasy Plus Mini-kit (Qiagen) and RNA was quantified and checked for its purity using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Reverse transcription was performed on 2 μg total RNA with the superscript III first-strand synthesis system for RT-PCR kit (Invitrogen) using a mixture of oligo (dT)20 and random hexamer primers. Primer sequences for conventional reference genes were obtained from [13] and primer sequences for the novel candidate reference genes proposed by RefGenes were designed using primer 3 software [33] (see list below). 20 ng total RNA equivalents of cDNA were used in each qPCR amplification run in triplicate. Detection of the PCR product was carried out by the LC480 real-time PCR detection system (Roche, Nutley, NJ, USA) using LightCycler 480 SYBR Green I Master mix and 250 nM primer. Relative quantities were calculated by the delta-Ct method and expression stability of the housekeeping genes was evaluated by GeNorm [13]. The primers used in this study were as follows:

Hs B2M F TGCTGTCTCCATGTTTGATGTATCT
Hs B2M R TCTCTGCTCCCCACCTCTAAGT
Hs GAPD F TGCACCACCAACTGCTTAGC
Hs GAPD R GGCATGGACTGTGGTCATGAG
Hs RPL13A F CCTGGAGGAGAAGAGGAAAGAGA
Hs RPL13A R TTGAGGACCTCTGTGTATTTGTCAA
Hs SDHA F TGGGAACAAGAGGGCATCTG
Hs SDHA R CCACCACTGCATCAAATTCATG
Hs YWHAZ F ACTTTTGGTACATTGTGGCTTCAA
Hs YWHAZ R CCGCCAGGACAAACCAGTAT
Hs BUD13 F GATGGAGATTTGCCTGTGGT
Hs BUD13 R ATTTGGCACTGGAACGAAAG
Hs EIF4EBP2 F TAGCCCTGGCACCTTAATTG
Hs EIF4EBP2 R AACTGAGCATCATCCCCAAC
Hs GOLT1B F CCTTATTGGTTGGCCTTTGA
Hs GOLT1B R AGCCAACAACGACAGGAAAG
Hs INTS4 F GCAGCTCCATGAAAGAGGAC
Hs INTS4 R ACCCAGATAAGCTGGACTGC
Hs SAP130 F GAGGCCAGTTTCTGCAGTTC
Hs SAP130 R GCACCAGGTGGTAGGTCACT
Hs TATDN2 F ACAAATGCTCTCCACCCCTA
Hs TATDN2 R TCCATCACCACCTCCCTATC
Hs ZNF410 F CTCCGAAAACATCTGGTGGT
Hs ZNF410 R CTGCAGGTGATGCTTTCTCA

RT-qPCR for cattle and pig liver

Immediately after slaughtering pieces of liver tissue were taken from calves and piglets fed different dietary fiber diets and snap frozen in liquid nitrogen. Total RNA was extracted with TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA quantity and quality were

assessed using a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) and a Bioanalyzer 2100 with RNA Nano Chips (Agilent Technologies, Palo Alto, CA). RNA integrity ranged between 7.2 and 8.4 and OD260/280 between 1.81 and 1.96. Samples were diluted to a working concentration of 10 ng/μL. Primers were chosen for cattle and pig orthologs of mouse genes identified as stably expressed in liver tissue. Primer design was done using the primer 3 software [26] and primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). One-step RT-qPCR (gene specific reverse transcription immediately followed by qPCR) was performed using SuperScirpt III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). PCR temperature profiles were optimized for each primer pair and identity of amplicons was verified by sequencing (Sequencing Service, Ludwig Maximilians Universitaet, Munich). Signal detection was achieved with a Rotor-Gene 3000 (Corbett Life Sciences, Sydney, Australia). Validation of the housekeeping genes was done by GenEx Professional Software ver. 4.4.2 (multiD Analyses AB, Gothenburg, Sweden) utilizing GeNorm and Normfinder. Below are the primers used for this study:

Bovine primers:

Bt ACTB F AACTCCATCATGAAGTGTGACG
Bt ACTB R GATCCACATCTGCTGGAAGG
Bt GAPDH F GTCTTCACTACCATGGAGAAGG
Bt GAPDH R TCATGGATGACCTTGGCCAG
Bt UBQ F AGATCCAGGATAAGGAAGGCAT
Bt UBQ R GCTCCACCTCCAGGGTGAT
Bt VPS4A F CAAAGCCAAGGAGAGCATTC
Bt VPS4A R ATGTTGGGCTTCTCCATCAC
Bt GAK F TCTGGGAAGTGGCAGAGAGT
Bt GAK R CGGCACGTCTGGTAGAAGAT
Bt PMPCA F CATCCCAGAATAAGTTTGGACAG
Bt PMPCA R AGAATCAGCAGACACAGCATACA

Porcine primers:

Ss UBIQ F AGATCCAGGATAAGGAAGGCAT
Ss UBIQ R GCTCCACCTCCAGGGTGAT
Ss Histon H3 F ACTGGCTACAAAAGCCGCTC
Ss Histon H3 R ACTTGCCTCCTGCAAAGCAC
Ss GAPDH F AGCAATGCCTCCTGTACCAC
Ss GAPDH R AAGCAGGGATGATGTTCTGG
Ss GAK F AATCGCAGTGATGTCCTTCC
Ss GAK R GCTTCGAGTCCAGAAACAGC
Ss VPS4A F CAAAGCCAAGGAGAGCATTC
Ss VPS4A R ATGTTGGGCTTCTCCATCAC
Ss PMPCA F CATCCCAGAATAAGTTTGGACAG
Ss PMPCA r AGAATCAGCAGACACAGCATACA

RT-qPCR for Arabidopsis tissues

Total RNA was isolated from 5 day old seedlings or from 15 day old leaves following the TRIzol protocol (Invitrogen). RNA quantity and quality was assayed via spectrophotometer analysis (Pharmacia Biotech). First-strand cDNA synthesis was performed with 3μg of total RNA using Superscript II RNase H- reverse transcriptase (Invitrogen) and oligo-dT primers (Fermentas) according to the manufacturer's instructions. The 20-μL cDNA reaction was diluted 1:100 with deionized water, and 4 μL were used for each RT-PCR amplification. Amplifications were performed as technical duplicates and biological quadruplicates in 96-well plates in a 20-μL reaction volume containing 10 μL 2x Fast SYBR Green qPCR MasterMix (Applied Biosystem). Reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). Primers for all amplifications, designed with PerlPrimer v1.1.10 (freeware by Owen Marshall), were located on exon-exon borders to prevent amplification of potentially contaminating genomic DNA.

Primers used for Arabidopsis seedlings:

At At3g24160 F ATATCAGACAGGCAGTCAGCG
AT At3g24160 R TGCTAAAGCATCGATACCACC
At At3g27820 F GCGGTGGCTATATCGGTATGG
At At3g27820 R AAAGAGACGTGCCATGCAGTG
At At1g13320 F CAAGTGAACCAGGTTATTGGGA
At At1g13320 R ATAGCCAGACGTACTCTCCAG

Primers used for Arabidopsis leaves:

At At3g61710 F AGACACAGGTTGAACAGCCA
At At3g61710 R GTATGCTTCCACGTCCCTCG
At At1g32050 F TCACCTACTTGATTCACATTGGCT
At At1g32050 R ATCAATTGCTGCAAGCACAC
At At3g01150 F CCACCGGAGCAGAGATTACAC
At At3g01150 R CAACTTTCTTGCCGTCAGCAC

Primers used for Arabidopsis shoot apices:

At At3G17920 F AACGACACTGTCAGATTCCA
At At3g17920 R CTACTTCCCGTTGCTTATAGGTG
At At2G17390 F CAGACTGTTGCAGCTGAACCT
At At2g17390 R GCTTTCAAACCCTCGACATCAC
At At5G51880 F CAGTATTGTAGCTGAGGTAGCTCC
At At5g51880 R CGCCTTTGGAGACATTCCTC

Authors contributions

TH, MW, SB, OL, WG and PZ elaborated the concepts, designed the tool, developed the software, and curated data. Validation experiments using RT-qPCR were performed with mouse liver samples by MD and PD, on cattle and pig liver by SM and MP, on Arabidopsis tissues by LB, and on human B-lymphocytes by PV and LK. All authors were involved in writing the manuscript.

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