Aus dem Institut für Tierernährung
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der Freien Universität Berlin

In vitro and *in vivo* studies on the effects of feed additives on a porcine intestinal
epithelial cell line (IPEC-J2) and in weaned piglets experimentally challenged with
enterotoxigenic *Escherichia coli*

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Contents

Abbreviations

ADG	average daily gain
ADF	acid detergent fiber
AGP	antimicrobial growth promoters
CFA	colonization factor antigen
ETEC	enterotoxigenic *Escherichia coli*
IPEC-J2	intestinal porcine epithelial cell line J2
NDF	neutral detergent fiber
NSP	non-starch polysaccharides
PWD	post weaning diarrhea
TEER	transepithelial electrical resistance
VFI	voluntary feed intake
VTEC	Verotoxin-producing *Escherichia coli*
YFP	yeast fermentation products

Tables

1. Introduction

The segregation from the sow constitutes a major challenge for growth, health and development of piglets. Natural weaning occurs over a period of several weeks at an age of around 70 days (Varley and Wiseman, 2001). As a result of this gradual process, milk as primary source of nourishment is replaced by solid feed. In modern rearing conditions this process is taking place abruptly at the age of 21-28 days whereby weaning at 21 days has more negative consequences on growth rate and stress endocrine responses than weaning at 28 days (Colson et al., 2006). The sudden separation from the sow and eventually from the littermates has an adverse effect on the piglet's general health and constitution and exposes piglets to both nutritional and environmental stress, resulting in reduced feed intake, growth retardation, digestive disorders and increased mortality. The transition to solid feed can result in a dramatically reduced voluntary feed intake in the first days following weaning. This situation causes a risk for atrophy of the intestinal villi and digestive disorders. Gene expression of pro-inflammatory cytokines is increased in this period (Pie et al., 2004) and piglets show elevated levels of blood and urine cortisol as well as suppressed lymphocyte function (Kanitz et al., 2002). Gastrointestinal disturbances include alterations in small intestinal architecture and enzyme activities. Weaning is frequently complicated by infections with various pathogens among which enterotoxigenic *Escherichia coli* (ETEC) play a major role. ETEC are often involved as causative agents in post weaning diarrhea (PWD) and cause significant losses among weaned piglets worldwide (Fairbrother et al., 2005). Antibiotic growth promoters (AGP) were known to increase performance and to protect piglets from post weaning diarrhea. The risk of development of bacterial resistance due to excessive use of AGPs in swine (Amezcua et al., 2002; Jensen et al., 2006a; Jensen et al., 2006b; Smith et al., 2010), growing consumers demand for environmental protection (Sarmah et al., 2006), as well as animal health and welfare and the concomitant risk for human health have forced authorities to ban the use of AGP within the European Union and to seek for alternatives pursuant to Regulation (EC) No. 1831/2003.

In this context, the present work investigated the effects of feed additives in a cell culture model (IPEC-J2) and in piglets after ETEC infection. An *in vitro* experiment

investigated the bacterial adhesion to a porcine intestinal cell line grown with or without the addition of different feed additives. Effects of feed additives on health and performance of piglets were tested in a feeding trial with an additional challenge with ETEC for induction of PWD.

2. Literature review

2.1 *Escherichia coli*

E. coli is a Gram-negative, rod-shaped bacterium with a typical diameter of 0.5 μm and a length of 2 μm, belonging to the family of Enterobacteriaceae. It was discovered in 1885 by the pediatrician Theodor Escherich (1857-1911) as *Bacterium coli commune* (Brock et al., 1994) and renamed in his honor in 1919. The organism is among the very first microbes to colonize the intestinal tract of neonatal humans and animals (Mackie et al., 1999). Here it benefits the host by production of vitamins (K2, B12) and prevention of establishment of pathogenic bacteria. Most *E. coli* strains are benign commensals of the intestine, that typically lack the virulence traits present in intestinal and extraintestinal pathogenic strains.

Prior to the identification of specific virulence factors, classification and analysis of the wide diversity of pathogenic *E. coli* strains was based on serological phenotyping, whereas it is now increasingly replaced by genotyping. The scheme proposed by Kauffmann in 1944 for the serologic classification of *E. coli* is however still used in modified form today. Kauffmann typed E. *coli* based on serologic identification of three major surface antigens: (1) the thermostabile lipopolysaccharide complexes (O antigens), that constitute part of the outer membrane, (2) the thermolabile flagellar antigen H and the capsular polysaccharide antigen K. More than 180 O, 60 H, and 80 K antigens have been proposed (Whitfield and Roberts, 1999; Robins-Browne and Hartland, 2002).

The bacterial compounds responsible for adhesion are now referred to as F for fimbrial antigens (Orskov et al., 1977). Many of these were previously known and recorded under different labels (for instance as K antigens in the Kauffman scheme). A new system designed for simplification of nomenclature was introduced in 1983 (Orskov and Orskov, 1983). According to this system colonization factor antigens (= fimbriae) previously referred to as CFA/I, CFA/II, K88, K99 and 987P were changed into F1, F2, F3, F4, F5 and F6, respectively (Krogfelt, 1991; Nagy and Fekete, 1999).

The bacterial O antigen defines the serogroup, a specific combination of O and H (and sometimes K) antigens defines the 'serotype' of an isolate (Kaper et al., 2004; Stenutz et al., 2006). The number of *E. coli* serotypes was estimated at 50.000 –

100.000 or more (Orskov and Orskov, 1992).The number of frequent pathogenic serotypes is, however, confined, e.g. only six pathotypes were found to account for 65.7% of 563 investigated isolates obtained from 507 pigs and 410 herds (Frydendahl, 2002). They belong to a limited number of clones, for which the O:K:H serotyping is a distinguished, although not faultless, marker.

Modern *E. coli* typing is increasingly based on molecular typing methods like DNA hybridization, pulsed-field gel electrophoresis or polymerase chain reaction (PCR). Here, gel based PCRs were soon replaced by more refined methods like real-time (Byun et al., 2013) and multiplex PCR (West et al., 2007; Bai et al., 2010) so that identification of multiple virulence factors is possible in a routine testing environment.

For accurate strain identification of pathogenic *E.coli,* today multilocus sequence typing (MLST) is considered the "gold standard" of molecular typing (Larsen et al., 2012).

The newly evolving Next Generation Sequencing has further improved throughput, scalability and speed of above mentioned genetic testing procedures and is about to transform genomic science (Schuster, 2007; Mardis, 2008). Rapid next-generation genomics technologies were successfully used to handle outbreaks of disease caused by pathogenic *E. coli* strains in humans (Mellmann et al., 2011).

This is increasingly important, since virulence factors of newly evolving strains are typically coded by mobile genetic elements (Dobrindt et al., 2003; Kaper et al., 2004; Bielaszewska et al., 2007). In this respect *E. coli* is increasingly recognized as a versatile and variable enterobacterial species, whose genome is not as fixed as originally thought (Bielaszewska et al., 2007). Serotypes that acquire accessory mobile genetic elements (plasmids, transposons, integrons and insertion-sequences) that code for virulence or invasion factors have high pathogenic potential. However, the presence of high numbers of *E. coli* cells harboring virulence genes does not necessarily correlate with disease and 68.8% of isolates obtained from clinically healthy pigs were positive for at least one virulence gene (Schierack et al., 2006b).

Overall an estimated 70% of post weaning losses in piglets is due to enteric *E. coli* infections (Jahn and Uecker, 1987).

Although generally considered to be more of an intestinal pathogen, *E.coli* has underrated significance as an extraintestinal pathogen as well (ExPEC) - with high pathogenic potential for causing extraintestinal disease in both animals and humans.

It is this diversity of a very versatile species, that makes accurate classification of all the different pathotypes difficult. This is not least due to the criteria applied for such a classification. When classified by their specific pathogenic mechanisms (toxins, adhesins, invasiveness, etc.) different pathogenic categories (pathotypes) may appear reasonable, than when classified on the basis of genetic or clinical criteria.

However, for intestinal pathogenic strains of *E.coli* 6 distinct pathotypes are recognized (Russo and Johnson, 2000): enterotoxigenic (ETEC), Shigatoxin producing/enterohemorrhagic (STEC/EHEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and diffusely adherent (DAEC).

With that being said, for weaner piglets ETEC are arguably the most significant *E. coli* and were isolated from 51 % of cases of PWD (Nakazawa et al., 1987).

2.1.1 Enterotoxigenic *Escherichia coli* and post weaning diarrhea

Due to its economic significance, the pathogenesis of the disease has been subject to comprehensive studies in the past years.

ETEC are characterized by non-destructive attachment to the microvilli that leaves the epithelium histologically normal with little or no signs of inflammation (Nagy and Fekete, 2005). ETEC are arguably the most significant *E. coli* pathotype for young swine and one of the most important porcine pathogens for the swine industry overall (Zhang et al., 2007). They are frequently involved as causative agents in PWD and cause significant losses among weaned piglets worldwide (Fairbrother et al., 2005). Infections of the gastrointestinal tract are the most serious problem that piglets face after weaning and ETEC diarrhea is the most common enteric disease in piglets, accounting for an estimated 50% of all losses (Gyles, 1994). Of the piglets that died because of PWD, 88.6% were infected with ETEC (Nagy et al., 1990).

ETEC involved in PWD typically produce alpha-hemolysin - 87.8% of 563 *E. coli* isolates from weaned pigs suffering from diarrhea were hemolytic (Frydendahl, 2002). The predominant serogroup of *E. coli* associated with PWD in pigs worldwide is O149 (Jamalludeen et al., 2007) followed by O8, O138, O139 and O141 (Frydendahl, 2002).

The key virulence factors of ETEC are fimbrial adhesins and enterotoxins. Their expression and combination strongly influence virulence characteristics (Nagy and Fekete, 1999).

Fimbrial adhesins or colonization factor antigens (CFA) mediate the colonization of the intestinal tract (Kaper et al., 2004). Pathogens bind with their fimbriae to specific receptors on the brush border of small intestinal enterocytes, thus preventing excretion due to intestinal peristalsis. Absence of specific receptors on the brush border causes resistance to ETEC infection (Sellwood et al., 1975; Francis, 2002).

The secreted heat-labile and heat-stable enterotoxins produced by bacteria after successful colonization act locally on the enterocytes. A net secretion of water and electrolytes results in secretory diarrhea, that is typically fluid and profuse (Kaper et al., 2004). Clinical symptoms like dehydration, exsiccosis, electrolyte imbalance (hyperkalemia, metabolic acidosis) and eventual circulatory shock are observed (Cox et al., 1991; Nagy and Fekete, 1999; Zajacova et al., 2013).

Virulence factors bring about three major advantages that pathogenic strains have over non-pathogenic ones. Diarrhea induced by toxins allows for greater dispersion of ETEC, fimbriae allow for adhesion and consequently greater reproduction in a suitable environment and greater numbers of bacteria provide greater opportunities for horizontal transport of virulence genes (Hagemann, 2006).

In addition to that, the dominance of ETEC has a detrimental effect on the natural *E. coli* diversity in the intestinal tract (Hinton et al., 1985) resulting in consequent suppression of other strains.

The severity of PWD is dependent on the concurrent influence of both infectious and non-infectious factors. PWD is a multifactorial disease (Melin et al., 2004) induced by various adverse factors impairing piglet health. Young weaning age, low hygienic status of the environment, unfavorable climate conditions or feed related factors modify the severity of the disease (Madec et al., 1998; Amadori et al., 2012; Campbell et al., 2013). Different combinations of these factors can lead to clinical conditions that are not uniformly defined. The following syndromes were induced in susceptible pigs through ETEC challenge: peracute fatal diarrhea; moderate diarrhea, weight loss and fecal shedding of the inoculum strain but also absence of clinical signs and shedding (Sarmiento et al., 1988).

2.1.2 Adhesins

Fimbriae are long filamentous polymeric surface proteins originating from the outer membrane of bacterial cells. They have a length of 0.5 – 1.5 µm, a molecular weight from 15 to 25 kDa and are peritrichously distributed in numbers of 100 – 300 (Ottow, 1975; Hedegaard and Klemm, 1989) per bacterium. Fimbriae of ETEC in animals (F4, F5, F6, F17, F18) are acquired virulence factors that are coded by plasmids (except F41) (Moseley et al., 1986). They are used by the bacterium to adhere to specific receptors (Blomberg et al., 1993) on the enterocytes and consequently colonize the intestine. Due to the receptor specificities of adhesins, ETEC strains seem to be quite host specific. The presence or absence of these specific receptors was found to be highly associated with increased fluid secretion as well as high fecal shedding of ETEC (Geenen et al., 2007; Niewold et al., 2007).

Fimbrial types are typically associated with certain O antigen types, like O149:F4 (Nagy et al., 1996); O139:F18ab or O141: F18ac (Nagy et al., 1997).

The fimbrial adhesins F18 and K88/F4 are encountered most frequently in young pigs with diarrhea (Osek et al., 1999; Francis, 2002; Frydendahl, 2002; de la Fe Rodriguez et al., 2011). Whereas F18 fimbriae are associated almost exclusively with diarrhea of pigs older than 3 weeks of age, F4 fimbriae are to be found in diarrhea of neonatal as well as weaned pigs (Wittig and Fabricius, 1992; Nagy and Fekete, 1999). Typically, ETEC carry only a single type of colonization pilus, but isolates carrying more than one fimbrium type exist (Nagy and Fekete, 1999; Chen et al., 2004). Fimbrial adhesins F4 and F18 occur in several antigenic forms.

In swine F4 fimbriae are the most common adhesin associated with diarrheal disease (Zhang et al., 2007). They exist in at least three variants: F4-ab, ac and ad (Guinée and Jansen, 1979). They share a common epitope "a", but express type-specific epitopes "b", "c" and "d". The variant F4ac is the dominant type and is mostly isolated from piglets affected by diarrhea (Westerman et al., 1988; Alexa et al., 2001; Francis, 2002).

Variants of F18 include F18ab and F18ac. F18ab is often associated with strains producing Stx2e, which causes edema disease, whereas F18ac is associated with ETEC causing PWD (Nagy et al., 1997).

F4 Fimbriae are composed of major and minor subunit structures. The binding site for the receptor of the porcine enterocytes is located on the major subunit. The minor

fimbrial subunits play an essential role in the biogenesis, but do not impact the adhesive properties of the F4 fimbriae (Bakker et al., 1992).

Adhesins are expressed only at 37 but not at 18°C, since the underlying genes are temperature-regulated (Fairbrother et al., 2005).

2.1.3 Enterotoxins

Enterotoxins are plasmid-regulated secreted proteins or peptides of ETEC bacteria that act on the intestinal epithelium. They occur as high-molecular-weight (88 kDa) heat-labile enterotoxins (LT) and low-molecular-weight (11 – 48 amino acid containing) heat-stable peptide toxins (ST). They produce profound functional changes in small intestinal epithelial cells by activation of intracellular second messenger pathways. It also appears that toxins can help overcome the innate mucosal barrier, which is a key step in enteric pathogen survival (Glenn et al., 2009).

Four enterotoxin genes have been reported in ETEC from pigs with PWD (Noamani et al., 2003) - elt (heat-labile enterotoxin, LT), estA (heat-stable enterotoxin STa or STI), estB (heat-stable enterotoxin STb or STII) and astA (enteroaggregative heat-stable enterotoxin, EAST1). Various combinations of elt, estA, and estB genes have been described in ETEC (Osek et al., 1999; Amezcua et al., 2002; Frydendahl, 2002).

LT is a complex hexameric AB5 toxin that shares immunological and structural properties with cholera toxin produced by *Vibrio cholerae*. The A subunit (LTA) is the toxic subunit that possesses enzymatic activity while the pentameric B subunits (LTB) facilitate attachment of the toxin to monosialoganglioside (GM1) on cellular membranes and subsequent uptake into the cell (Dallas and Falkow, 1980; Fekete et al., 2013). Heat-labile toxins are classified into two serological groups (LT-I and LT-II). While LT-I is neutralized by antiserum against cholera toxin (Pickett et al., 1986), LT-II is not (Green et al., 1983). LT increases intracellular cAMP concentrations, which raises the intracellular Ca^{2+} level and leads to Cl^- secretion.

STs are small polypeptides (2,000 Da) with low immunogenic effects. In contrast to LT, ST peptides remain active even after 30 min of boiling at 100 °C (Hirayama and Wada, 2000).

STa is soluble in methanol and protease resistant. It is a 18 or 19 amino acid cysteine-rich peptide, that exerts its effect by binding and subsequent activation of intestinal guanylyl cyclase C (GC-C) (Schulz et al., 1990; Schulz et al., 2003), causing an increase of the intracellular concentration of cGMP, which leads to Cl^- secretion and diarrhea.

STb is methanol insoluble and protease sensitive. It is made of 48 amino acids and increases intracellular levels of Ca^{2+} (Dreyfus et al., 1993).

EAST1 is a 38-amino acid protein featuring four cysteine residues. It shares some structural and functional similarities with STa enterotoxin (i.e. disulfide bonds, activation of guanylate cyclase in the intestinal epithelial cells). Although it was originally isolated from enteroaggregative E. *coli* (EAEC) (Menard and Dubreuil, 2002), EAST1 has been detected in other pathogenic *E. coli* groups as well. The most common EAST1-positive *E. coli* serotype was O149:K91 (mostly LTI/STII-positive). A close correlation between the presence of the EAST1 gene and F4 fimbriae was observed (Osek, 2003). The EAST1 toxin alone was not able to induce diarrhea in gnotobiotic piglets (Ruan et al., 2012; Zajacova et al., 2013). Its precise role in PWD of pigs remains to be elucidated.

A less understood virulence factor of ETEC is a 100-kDa adhesin involved in diffuse adherence (AIDA). It was recently shown to be associated with ETEC and VTEC virulence factors (Niewerth et al., 2001; Ngeleka, 2002). AIDA is assembled via a preliminary precursor protein, which has to be cleaved for correct maturation of the final toxin (Benz and Schmidt, 1993). Although AIDA was originally found on human EPEC strains (Benz and Schmidt, 1992), it occurs much more frequently in porcine than it does in human or other mammalian *E. coli* isolates (Niewerth et al., 2001). AIDA is understood to play more of a contributory role - its pathogenic function in PWD is still not fully examined.

2.1.4 Challenge models in piglets with enterotoxigenic *Escherichia coli*

In order to conduct further studies on this complex and significant disease, it is important to have a reproducible model of *E. coli* induced PWD available. It is mandatory to standardize the conditions of challenge experiments as far as possible, since the hygienic conditions in research facilities are very unlikely to adequately reflect in-field conditions (Sørensen et al., 2009).

However, there have been difficulties in experimental reproduction of the disease under experimental conditions (Chandler and Mynott, 1998). Challenging healthy piglets with ETEC alone led to a more subclinical form of PWD (Opapeju, 2009), especially when weaning age was increased (Wellock et al., 2007). In this context, ETEC are more and more understood to be only one of the essential components in the etiology of PWD (Nagy and Fekete, 2005).

A cofactor besides ETEC infection was found to be necessary in experimental studies aiming at inducing diarrhea and hypovolemia in newly-weaned piglets (Cox et al., 1991). The various procedures comprised administration of ETEC bacteria combined with previous antibiotic pretreatment, co-infection with transmissible gastroenteritis virus (TGEV), use of sodium bicarbonate as well as administration of toxins alone.

The etiology of PWD as a multifactorial disease in which ETEC are considered to be "the final link in a chain of complex biological processes" was also confirmed by comprehensive studies to reproduce the disease experimentally (Madec et al., 2000). A wide variety of factors was considered, incl. use of different ETEC strains and doses, time frame of inoculation, feed deprivation prior to infection as well as application of bicarbonate and testing for F4 receptor susceptibility (see table 1).

An experimental model to induce PWD using as few additional stress factors as possible was successfully introduced (Melin et al., 2004). Pretreatment with antibiotics and infection via gastric tube were avoided. Instead, piglets were infected with ETEC from up to 3 different serogroups and adrenocorticotropic hormone (ACTH) was used to investigate the effect of stress on susceptibility to ETEC. Infection was designed to emulate the natural exposition of animals to the pathogen. Therefore infection broth was spread to a density of 2×10^6 colony forming units of each strain per square meter on the floor of pens.

A Verotoxin 2e (VT2e) producing strain used for experimental infection caused diarrhea in 96.7% of all piglets (Rossi et al., 2012). However, also in this study a high protein content in the diet, infection immediately after weaning as well as bicarbonate application were used as additional stressors.

Table 1: Overview on ETEC challenge trials in piglets

Reference	ETEC strain	Toxins	Dose of infection, cfu	Amount of piglets	Weaning age, days	Adapt. time, days	Duration, days	Dietary factor	Additional stressors	Induction of PWD	Protein sources in %	Crude protein in %	Dietary crude fiber in %	Remarks
Jiang et al. (2015)	O149:F4	LT STb	1×10^9 (in 4 ml)	192	24 ± 2	8	35	essent. oils + enzyme combination	ns*	significant increase in fecal score	herring meal soy (amounts vary)	19,3	3,2	▪diets met 2012 NRC[1] requirements
Rong et al. (2015)	K88ac	LT STb	1×10^9 (in 30 ml)	18	23	8	12	Casein glycomacro-peptide	NaCHO$_3$ + sucrose a.i.	mild diarrhea; ADFI & ADG reduced	whey 15.0 soy meal 6.5 fish meal 6.0	19.0	1.77	▪diets met 2012 NRC specifications
Sugiharto et al. (2015)	O149:F4	ns	5×10^7 (in 20 ml)	52	30 ± 2	1	11	whey permeate / lactic acid bacteria	2 inf. (d 2&3) via orogastric tube	ns	ns	ns	ns	▪F4 suscept. verified a.i.[2]
Kwon et al. (2014)	K88	ns	3×10^{10} (in 3 ml)	32	28	7	14	coated zinc oxide	ns	mild diarrhea	soy, whey (amounts vary)	16.5	ns	▪only castrated male piglets
Ren et al. (2014)	K88	ns	10 ml (1×10^9/ml)	36	21	18	20	threonine	ns	ns	corn 54 peanut 20 whey 5	16.2	ns	▪25-27 °C for 2 d ▪amino acids met 2012 NRC requirements
Verhelst et al. (2014)	0149:K91: K88ac (CVI-1000)	LT+ STb+	5 ml (1×10^9/ml)	40	21	6	16	polyphenol extracts	24°C; fasting post inf.; 2 inf. (d 6&7)	yes	ns	ns	ns	▪tested a.i. for absence of ETEC ▪F4 receptor status determined post mort.
Kiarie et al. (2012)	K88	LT ST	6 ml (5×10^{10}/ml)	102	21	7	7	yeast ferment. product	ns	ns	soy 20.7 whey 20 fishmeal 5	21.3-21.6	ns	▪a.i. ETEC not detectable ▪diets met 1998 NRC specifications
Hedemann & Knud-sen (2010)	O149:F4	LT,STb EAST1	1×10^8 (in 20 ml)	48 (F4+)	49	2	22	chicoree	2 inf. (d 2&3) via stomach tube	moderate	fishmeal 12.5 soy 6.7 potato 5.5	23.9-24.8	13.1-13.3	▪creep feed d 14-39 of life

Reference	ETEC strain	Toxins	Dose of infection, cfu	Amount of piglets	Weaning age, days	Adapt. time, days	Duration, days	Dietary factor	Additional stressors	Induction of PWD	Protein sources in %	Crude protein in %	Dietary crude fiber in %	Remarks
Jansman et al. (2010)	O149: K91: K88ac	LT Stb	5ml (1x10^9/ml)	72	28	6	22	pea	ns	mild diarrhea	soy, potato protein, pea (amounts vary)	ns	ns	▪diets formulated to be nutritionally adequate (CVB[3], 2005)
Krause et al. (2010)	3 K88 strains (2-12,I-36, B104)	ns	6 ml (2 of each strain- 2,3x10^9/ml)	40	17	7	17	pro- & prebiotics (raw potato starch)	inf. with 3 different strains	mild diarrhea	soy 32.8 /39.0 whey 12 fishmeal 1.0/3.0	21.7-21.9	2.3 & 2.5	/
Molist et al. (2010)	K88	ns	6ml (2,2x10^{10}/ml)	36	17	9	14	wheat bran	ns	mild diarrhea	corn 32 soy 14	20.9	ns	▪diets met 1998 NRC specifications
Verdonk et al. (2010)	O149: K91:K88	LT+	2x10 ml (1x10^9/ml)	36	26	ns	14	soy	d 1-5 AGP via drinking water	moderate	whey 6.5 soy 16-17.5 potato 3.5/6.6	20.3-20.9	ns	/
Zhang et al. (2010)	O149: K91: K88ac	ns	10ml (1x10^9 cfu/ml)	18	18	7	14	*Lactobacill. rhamnosus*	ns	yes	ns	22.3	ns	▪no AGP ▪creep feed from d7 ▪"standard weaner diet"
Halas et al. (2009)	O149: K91:K88	LT Sta Stb	d3:3x10^7 d4:2x10^9 d5:1x10^{10} d6:5x10^8	48	21 ± 3	3	21	inulin & benzoic acid	four infections	moderate	soy 15 fishmeal 7.5/10 whey 5	22.1 & 22.0	ADF:2.4-2.7 NDF:7.2-8.2 total fibre: 9.5 - 10.6	▪no ETEC shedding at weaning ▪no dietary stress
Heo et al. (2009)	O149: K91:K88	LT Sta Stb	3/8/8 ml on d 3,4,5 (1x10^7/ml)	72	21	3	28	protein	3 infections (d 3,4&5)	moderate "subclinical"	soy 3.9 /20 fishmeal 2.2 /5.3 whey 5 /8.1	18 & 24	3.0 & 3.5	▪no AGP in diet ▪ a.i. piglets free of haemolyt. *E.coli* ▪ideal aa[4] pattern

Reference	ETEC strain	Toxins	Dose of infection, cfu	Amount of piglets	Weaning age, days	Adapt. time, days	Duration, days	Dietary factor	Additional stressors	Induction of PWD	Protein sources in %	Crude protein in %	Dietary crude fiber in %	Remarks
Jamallu-deen et al. (2009)	O149: H10:F4	Sta STb LT EAST-I	1x10^10 (in 5 ml)	101 (F4+)	21	2	6	bacterio-phages	Florfenicol for 2d + NaHCO$_3$ 15 min a.i.	yes	ns	ns	ns	▪a.i. test for haemolyt. O149 ETEC ▪F4 suscept. verified
Opapeju et al. (2009)	K88	LT STb	6 ml (5x10^10/ml)	40	17 ± 1	7	14	protein	inf. by gavage	mild diarrhea "subclinical"	soy 4 fishmeal 6 whey 7 plasma 2 casein 0/7.9	17.6 & 22.5	total NSP 7.7 & 8.0	▪a.i. piglets free from Ciprofloxacin resistant ETEC
Trevisi et al. (2009)	O149: K88ac	ns	1x10^10 (in 1.5 ml)	64	21	4	23	protein (aa: Trp.)	Colistin d 1-4 pw	mild diarrhea	soy 21 whey 8.5	18.7	2.8	▪testing for ETEC suscept. (in vitro villus adhesion assay)
Sørensen et al. (2008)	O149: F4ac	LT STb EAST1	1x10^8	128 (F4+)	49 (organic product.)	1	10	feed & prot. restriction, lupin, Vit.E	inf. by gavage (d 1,2&3 pw)	mostly yes	fishmeal, soy, peas, rape, potato protein (amounts vary)	12-20.1	ns	▪creep feed from 14 d of age
Wellock et al. (2007)	O149: K91:K88	ns	1x10^9 (in 10 ml)	64	27	3	14	non-starch polysacch. solubility	ns	no	herring meal 10 milkpowder 12,5 full fat soya 7 whey 1.3-6.9	22.8 - 24.0	total NSP + fructan 9.5 - 16.9	▪F4-suscept. unknown ▪a.i. ETEC free ▪creep feed for last 2 weeks of suckling
Owusu-Asiedu et al. (2003)	K88	ns	6 ml (1x10^10/ml)	96	10	6	14	plasma/ pea prot.+egg yolk, ZnO, fumar. acid, antibiotic	ns	mainly mild diarrhea	whey 13 soy 16 fishmeal 8 (+pea 5 /10 or plasma 5 /10)	26.2 - 26.5	ns	/
Van Dijk et al. (2001)	O139: K82	LT-	1x10^10 (in 2x10 ml)	20	19	6	14	porcine plasma	24°C; Colistin d 1-5 pw; 2 time inf.	yes (65% mortality)	soy, whey, (porcine plasma)	16.1-17.4	2.5 & 2.6	▪ piglets fasted on d 1&2 post weaning

*ns not specified [1]NRC National Research Council [2]a.i. ante infectionem [3]CVB central veevoederbureau (Central Feedstuff Bureau) [4]amino acid

2.1.4 Effects of various feed additives *in vitro*

Since the use of AGPs was banned in the EU in 2003 (Regulation (EC) No. 1831/2003), research for alternatives to improve piglet health after weaning became a high priority. However, animal testing is demanding and causes issues on animal welfare. Cell culture is considered as alternative to partly replace *in vivo* experiments. The use of human and mouse cell lines was shown to be inappropriate to investigate ETEC pathogenesis in swine (Mariani et al., 2009).

Shiga toxin-producing *E. coli* (STEC) strains showed very host-specific patterns of interaction on human intestinal epithelial cell lines T84 and HCT-8 compared with the porcine cell line IPEC-J2 (Sonntag et al., 2005), rendering them unsuitable for use in swine experiments. To our knowledge, only three porcine intestinal epithelial cell lines have been characterized: IPEC-1 (Gonzalez-Vallina et al., 1996), IPEC-J2 (Schierack et al., 2006a) and the transformed and immortalized IPI-2I cell line (Kaeffer et al., 1993).

Various studies were performed to investigate the interaction of nutritional factors with *E. coli* adhesion and cell integrity in IPEC-1 cells. *Lactobacillus sobrius*, a probiotic strain isolated from the pig intestine, reduced ETEC adhesion as well as membrane damage in IPEC-1 cells (Roselli et al., 2007b). Several feed ingredients and additives, including bovine colostrum, bromelain and yeast extract as well as daidzein and allicin prevented a decrease of transepithelial electrical resistance (TEER) in IPEC-1 cells after infection with an ETEC strain (Roselli et al., 2007a).

The IPEC-J2 cell line has emerged as the most relevant porcine cell line for *in vitro* challenge studies with various enteric pathogens (Schmidt et al., 2008). It was derived from small intestinal tissue of a neonatal, unsuckled piglet (Berschneider, 1989) and has been characterized extensively (Schierack et al., 2006a; Koh et al., 2007; Brosnahan and Brown, 2012). Differentiation to a single monolayer of polarized enterocyte-like cells with apical microvilli, junctional complexes, intercellular spaces and tight junctions (Geens and Niewold, 2011) was observed. These data allow for positive validation of IPEC-J2 cells as *in vitro* model of the porcine intestine, both electrophysiologically and morphologically.

It has also been extensively used to investigate factors associated with adhesion of pathogenic *E. coli* in the porcine intestine. This adhesion of ETEC to IPEC-J2 cells is strain specific (Koh et al., 2007) and substantially determined by the presence of flagella and fimbriae. This was shown for F18ab positive *E. coli* (Duan et al., 2012) as well as for F4ac positive ETEC (Zhou et al., 2013). IPEC-J2 most likely express a F4 receptor, which was suggested to be the reason why a F4 positive strain had a considerably more severe impact on IPEC-J2 cells than an identical strain not expressing the F4 fimbrium, causing a decrease in TEER and higher IL-8 expression (Geens and Niewold, 2010). Confirming this, a fimbriated ETEC strain was markedly more effective than a non-fimbriated one at adherence to IPEC-J2 cells and inducing an inflammatory response (Hermes et al., 2011).

IPEC-J2 cells have also been used to characterize the contribution of LT to the adhesion of *E. coli* to epithelial cells. The presence of the heat labile enterotoxin was shown to have a positive impact on adhesion to IPEC-J2 cells (Johnson et al., 2009; Fekete et al., 2013). Carrageenan, a sulfated polysaccharide mimicking epithelial receptor structure, was however able to stabilize membranes when heat-stable toxin b (STb) was applied to IPEC-J2 cells (Goncalves et al., 2008).

Zinc oxide modulated the inflammatory and metabolic response of IPEC-J2 cells infected with ETEC (Sargeant et al., 2010; Sargeant et al., 2011).
Extracts from wheat bran, casein glycomacropeptide, mannan-oligosaccharides, locust bean extract and a fermentation product from Aspergillus oryzae reduced *E. coli* attachment to IPEC-J2 cells (Hermes et al., 2011).
IPEC-J2 cells have also been successfully used to establish screening methods to characterize adhesion capabilities of various *E. coli* strains (Schierack et al., 2013).

2.1.5 Effects of various feed components and additives *in vivo*

Despite significant progress in the field of *in vitro* cell lines, experiments in living animals have remained indispensable for acquiring reliable information about effects of investigated feed additives.

A wide range of feed compounds, additives and natural substances have been tested for this matter and many have shown positive impact when fed to piglets challenged with ETEC.

Carbohydrates like wheat bran, fed at 4% of the diet, reduced intestinal ETEC numbers (Molist et al., 2010). Inulin fed at high levels (8%) of the diet reduced the incidence of PWD and improved fecal scores (Halas et al., 2009).

Functional proteins like porcine plasma included at 10% (Owusu-Asiedu et al., 2002), or 8% respectively (van Dijk et al., 2002) into the diet improved fecal scores and overall appearance and performance of infected piglets.

So-called pharmacological doses of zinc oxide also affect clinical outcome and the intestinal microbiota (Holm and Poulsen, 1996; Owusu-Asiedu et al., 2003; Heo et al., 2010; Vahjen et al., 2011). Positive results have also been observed when feeding non-organic clay compounds (Trckova et al., 2009).

A detailed review about possible feeding strategies to replace AGP was provided (Heo et al., 2013).

2.2 Previous experience with used feed additives

In addition to the above-mentioned substances, research has focused on feed components, which are isolated from natural sources or are considered as "natural" substances.

Bovine colostrum (CM) is a compound being especially rich in specific immunoglobulins as well as various other non-specific compounds with antimicrobial and antiviral activity like lactoferrin, lactoperoxidase or lysozyme (van Hooijdonk et al., 2000). Bovine colostrum included at a concentration of 4% in the diet was found to reduce growth check and diarrheal episodes in non-challenged weaned piglets (Huguet et al., 2012). Bovine colostrum also triggered a short-lived systemic immune response measured by increased IgA concentrations (Boudry et al., 2008).

Bromelain is a mixture of proteolytic enzymes extracted from the juice and stems of pineapple. Piglets that were orally administered 12.5 and 125 mg of Bromelain respectively, had less post weaning diarrhea and increased weight gain compared with untreated animals after ETEC challenge (Chandler and Mynott, 1998). This was

presumably because of temporary modification of the ETEC receptor, which is thought to be sensitive to enzymatic treatment. Previously it was already shown, that oral administration of this protease inhibited F4 ETEC attachment to the porcine small intestine in a dose dependent manner (Mynott et al., 1996).

Yeast preparations from *Saccharomyces cerevisiae* had a positive impact in piglets when sows were fed a diet with yeast as well. They exhibited increased postweaning weight gain and improved feed efficiency compared with piglets not receiving yeast (Jurgens et al., 1997). While only minimal effects on intestinal health were observed in early-weaned piglets (White et al., 2002), yeast fermentation products improved growth performance, indices of immunity and intestinal health in weanling pigs (van der Peet-Schwering et al., 2007). Yeast fermentation products from *Saccharomyces cerevisiae* exerted some degree of gut protection and decreased fecal shedding in piglets challenged with ETEC (Kiarie et al., 2011).

Acidifying compounds can exert antimicrobial effects by compensating the inability of the piglet to secrete sufficient amounts of acid in the stomach after weaning (Risley et al., 1992). Maintaining appropriate pH after weaning secures efficient digestion of protein as well as elimination of pathogens. Organic acids were found to serve as alternatives for antimicrobial growth promoters (Partanen and Mroz, 1999). Acidification through feeding of fumaric acid reduced the incidence and severity of diarrhea after ETEC infection (Owusu-Asiedu et al., 2003).

Thyme essential oil showed an inhibitory activity against E. *coli* O157:H7 in several *in vitro* studies, for instance in minced beef meat (Solomakos et al., 2008). Feeding trials (with concentrations 0.1%, 0.5% and 1%) did not reveal any effects on *E. coli* in the gut of weanling piglets (Hagmüller et al., 2006). Although thyme showed antibacterial potential *in vitro* against 39 hemolytic *E. coli* strains this could not be confirmed in feeding trials with piglets (Jugl-Chizzola et al., 2005).

2.3 Aims

Based on the literature data, five different additives were used in different concentrations to test for protective effects on IPEC-J2 cells against ETEC infection. We hypothesized that treatment with bovine colostrum, pineapple stem extract containing bromelain, an autolysed yeast preparation (*Saccharomyces cerevisiae*), a combination of organic acids or the essential oil from thyme may limit adhesion of ETEC to IPEC-J2 cells. Bacterial adhesion to IPEC-J2 cells was measured using flow cytometry. Further on, it was hypothesized that feeding the aforementioned additives may limit diarrheal episodes and improve overall performance in piglets.

3. Effect of different feed ingredients and additives on IPEC-J2 cells challenged with an enterotoxigenic *Escherichia coli* strain

4. A standardised challenge model with an enterotoxigenic F4+ *Escherichia coli* strain in piglets assessing clinical traits and faecal shedding of *fae* and *est-II* toxin genes

5. Discussion

It was the aim of this study to investigate potential effects of feed additives on ETEC adhesion and pathogenicity in porcine epithelial cells (IPEC-J2) and in a challenge experiment with piglets. Diarrheal disorders are a major problem for weaned piglets. Growing societal demands for health and wellbeing of farm animals and safe food of animal origin has initiated a broad spectrum of research activities. In this respect it is of importance to find active compounds, which have reliable protective effects in the post weaning period. The growing demand for alternatives to antibiotic treatments has let to extensive research and testing of various "natural" substances. After the effect of feed additives on ETEC infection was tested in a suitable *in vitro* cell culture, a challenge model was established, that investigated the effect of feed additives on piglet health under ETEC challenge in a feeding trial.

5.1 Methodological aspects

5.1.1 Preparation of feed additives for *in vitro* use

When considering the results of the different feed additives in the present study, it has to be taken into account, that powdered and pulverized feed additives needed to be sterilized before use in the *in vitro* experiment. Thermal sterilization would have been an option, but was not used due to the risk of destroying the intrinsic structure of the feed additives. The aqueous extracts were therefore sterile-filtered. Additive fractions are differently soluble in aqueous media, and thus an unknown fraction of the substrates was either withheld in the filter or not dissolved. Consequently, the results for the tested feed additives relate to the filtered water-soluble fraction only and might therefore deviate from the additives effect when included in their entirety. The active compounds of the additives were not identified since this was not subject of the study.

5.1.2 Suitability of IPEC-J2 cells as a model of the porcine intestine

A porcine intestinal epithelial cell line was cultivated under standard conditions to study the effect of feed additives on the adhesion of pathogenic ETEC. The IPEC-J2 cell line has proven its suitability as an *in vitro* intestinal model (Schierack et al., 2006a; Awad et al., 2011; Brosnahan and Brown, 2012). Because of its manifold

electrophysiological and morphological functions and cell characteristics including single monolayer of polarized enterocyte-like cells with apical microvilli, junctional complexes, intercellular spaces and tight junctions (Geens and Niewold, 2011) it is now established as a suitable *in vitro* model of the porcine intestine and has emerged as the most relevant porcine cell line for *in vitro* challenge studies with various enteric pathogens (Koh et al., 2007; Schmidt et al., 2008; Gommel et al., 2013; Lan et al., 2013; Zhou et al., 2013).

The arguably most important feature of the IPEC-J2 cell line in respect to ETEC studies is the highly probable presence of F4 receptors (Geens and Niewold, 2010). A F4 positive strain was markedly better at adhering to IPEC-J2 cells and inducing an inflammatory response (Hermes et al., 2011) as well as causing both a decrease in TEER and higher IL-8 expression (Geens and Niewold, 2010) than a non fimbriated strain.

To detect bacteria and investigate adhesion to epithelial cells in flow cytometry, bacteria were labeled with a fluorescent dye beforehand. Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) is a non-fluorescent membrane permeable ester that is converted to a fluorescent molecule by non-specific intracellular esterases. An increase of fluorescence intensity of epithelial cells can be ascribed to bacterial adhesion. The same design was already successfully used to investigate adhesion of *Streptococcus pyogenes* (Sethman et al., 2002; Hytonen et al., 2006) or *Helicobacter pylori* (Logan et al., 1998) with epithelial host cells.

However, it can be argued that genetic modification to express fluorescent proteins or direct labeling with fluorochromes prior to infection could introduce a bias when measuring fine differences in adhesion of pathogens. Responding to that concern proposals using post-adhesion and post-invasion processing of bacteria have been made (Trouillet et al., 2011).

5.1.3 Challenge trial measurements and assessment of scores

To collect information about the course of this multifaceted disease health status was assessed using various methods.

Overall health status was assessed directly on a daily basis by general condition score, conduct score and body temperature. Scores were ascribed subjectively upon

examination on a daily basis while body temperature was measured rectally from day 1 before until day 7 after infection using an electronic thermometer. Those parameters were considered to reflect the general constitution and state of health of the animals.

Similarly, the conduct score was performed to describe general behavior patterns.

A drop in body temperature was frequently observed during diarrheal episodes, even if symptoms were blurred or short-lived. Except for peracute cases, all casualties showed a significant drop in body temperature prior to death.

Feed intake gave additional information - a reduction in feed intake for about 3-4 days after infection is a good indicator that challenge was effective. In that time frame non-infected control animals consumed almost twice as much feed as infected animals. For reasons of animal welfare piglets were held in pairs of two animals per pen. The feed intake was therefore ascertained per pen and half of it was apportioned to each piglet. The inevitably inherent error of this procedure could not be averted.

The fecal characteristics were assessed on a daily basis by a standardized fecal scoring and the determination of fecal dry matter content. Fecal dry matter content was determined from samples taken daily from day 1 before until day 7 after infection. This time frame was considered appropriate since in prior trials diarrhea usually lasted no longer than 3-4 days after infection (Owusu-Asiedu et al., 2003; Zhang et al., 2010). Additionally, dry matter served as an objective evaluation of the bias possibly inherent in the assessment of fecal scores.

5.1.4 Feed Additives

Feed additives were taken up by piglets via feed, however, the amount differed individually. It has to be taken into account that limited feed intake at the beginning of the trial also entailed little effective uptake of feed additives. The dose of respective feed additive present in the bowel of a piglet at any particular time might have been variable and in case of low feed intakes insignificant. Direct application or offering the feed additive as top dressing should be considered as an alternative, but was not considered to reflect the practical situation. No clear statement about the individual amount consumed can be made.

5.2 Discussion of the Results

5.2.1 Effect of feed additives on adhesion of enterotoxigenic *Escherichia coli in vitro*

Cells were grown in plain cell medium and in cell medium containing four different concentrations of the tested feed additives. Before infection mean intrinsic fluorescence was equal among all treatments (p=0.778 and p=0.93). No clear opinion can be given on the effect of bromelain containing pineapple stem extract, since this substance lysed IPEC-J2 cells until dilution of 10^{-4} and made testing impossible. Subsequent infection showed that Acid mix and bovine colostrum seemed to favor bacterial adhesion to IPEC-J2 cells leading to increased values of fluorescence. Numerically positive results were observed with thyme extracts. Thyme appeared to show a concentration dependant effect, since fluorescence of infected epithelial cells decreased with higher dilution.

Only the yeast preparation from *Saccharomyces cerevisiae* was able to inhibit bacterial adhesion in a statistically significant way. Fluorescence was decreased by almost 50 % in the two highest dilutions. After infection only 2.2 and 8.1 %, respectively, of cells treated with one of those two dilutions of the yeast preparation PV were showing fluorescence intensities, that would indicate ETEC adhesion (the standard intrinsic fluorescence was taken as a reference value). The ability of yeast preparations to prevent ETEC adhesion to IPEC-J2 cells had been encountered previously (van der Aa Kühle et al., 2005).

5.2.2. Effect of ETEC challenge on performance and health parameters of piglets

5.2.2.1 General condition, behavior and body temperature

The disease manifested in variable and inconsistent patterns. Both, severity and duration of disease were subject to pronounced variation.

All piglets were affected and showed different symptoms in the aftermath of infection, ranging from disturbed general condition, reduced expression of species-specific exploratory behavior, reduced attentiveness and flight behavior through to apathy. Within 24 h after infection all piglets were displaying condition scores of 2-3 starting from an initial score of 1. In the acute phase of infection, non-infected animals were

significantly different in both conduct (p=0.007) and condition score (p=0.010) from infected animals. Mortality amounted to 16% among infected piglets which is congruent with findings in other studies. At the same time there were no losses and no signs of disease in the non-infected control group.

5.2.2.2 Fecal characteristics

The majority (82 %) of infected piglets showed distinct drop in fecal dry matter at some point after infection. There were no statistically different results in fecal score and dry matter content except between bromelain treated animals and the non-infected control group. During the infection period, bromelain treated piglets had the lowest dry matter contents whereas colostrum and PV treated piglets had the highest fecal scores and highest dry matter contents. However, this was not statistically significant except between bromelain treated piglets and the non-infected control group (p=0.024). This suggests that bromelain did not have the ability to affect the occurrence of diarrhea under the given conditions.

Fecal characteristics were usually returning to normal within 3 days after challenge. The longer animals suffered from reduced fecal dry matter the more body temperature tended to drop, which was usually accompanied by reduced feed intake. The risk of mortality was increased under these conditions.

5.2.2.3 Body weight, feed intake and feed conversion

At day of weaning, non-infected control group animals had slightly lower body weight (5.2 kg) than the piglets in the infected groups (5.7 kg). However at day 14, average body weight of infected animal was 8.5 kg compared to 8.7 kg in the non-infected control group. The impact of the infection was clearly reflected in reduced feed intake. For about 3-4 days after infection feed intake stagnated and a temporary deterioration of health status occurred. None of the feed additives was able to prevent this health affection. During the first 3 days after infection non infected control piglets consumed more than twice as much feed (192 g) compared with infected animals (91 g) (p=0.064). Infected piglets ingesting diets with Acid mix (401 g) and Thyme (386 g) were able to keep up a feed intake equal to that of non-infected control animals (391 g) in the aftermath of infection, while piglets fed with the other feed additives consumed markedly less feed per day in that time frame (ca. 296 g).

Consequently Acid Mix (9.3 kg) and Thyme (8.9 kg) fed piglets eventually outweighed non-infected control piglets (8.7 kg) at the end of the trial at day 14 after infection. The suspected efficiency of these two feed additives was substantiated by the average daily gain (ADG). Over the course of the whole experiment the group being fed with Acid mix (218 g/d) was the only to gain more weight per day than non-infected control piglets (203 g/d), while the group receiving the diet with Thyme (193 g/d) was nearly equal. Although these findings were not statistically significant, it might hint towards a potential of those two feed additives to increase feed uptake and thus sustain adequate body weight development.

Low birth or weaning weight did not automatically predispose piglets to develop PWD. Suffering from diarrhea did not necessarily lead to loss in body weight and some animals were able to make up for lost nutrients by sustaining an appropriate feed intake. On the other hand some animals also reduced feed intake while not expressing diarrhea or other clinical signs of disease. Results were inconsistent and difficult to interpret.

5.2.2.4 Quantification of fecal shedding of *fae* and *est-II* virulence genes

Feed additives did not have a significant effect on the shedding of virulence factors. Altogether the shedding of the tested toxin genes was evenly distributed among infected animals, individual variations were however considerable. The fecal concentrations of the virulence factor genes *fae* (F4ac fimbrium) and *est-II* (heat stable toxin Stb) of the challenge strain were measured by quantitative real-time PCR in samples that were rectally taken one day before and 3 and 7 days after challenge. The virulence factor gene *est-II* was already present in the population, suggesting a previous contact of piglets with *E. coli* strains carrying that gene or indicating that strains carrying this gene are part of the indigenous gut flora of pigs (Schierack et al., 2006b). Since the *fae* gene was not detectable before the challenge, it can be assumed that the experimental animals were not in prior contact with the challenge strain. The virulence genes of the challenge strain were detectable in high amounts at day 3 after challenge. However, one or both virulence genes were not detectable in 5 out of 84 piglets at day 3 after challenge. At day 7 shedding had already abated markedly, but was still above pre-infection level. This is in congruence with former study results, which found no excretion of ETEC at day 10 after infection (Kiers et al., 2006).

Interestingly, the amount of virulence gene shedding in the non-infected control group increased over the course of the experiment. This was despite the considerable effort that had been made to prevent horizontal spread of bacteria, including separate housing, disinfection, change of shoes, protective clothing and the sequence of examination (Rossi et al., 2012).

5.2.3 Comparison of the challenge model with in field conditions

The change of the enteric flora after weaning enables colonization with ETEC (Konstantinov et al., 2006) and subsequent development of PWD in piglets. Environmental factors around weaning have a great influence on the pathogenesis of the disease. Post weaning digestive disorders could be prevented to a large extent, if zootechnical conditions were controlled (Madec et al., 1998). It can be argued that conditions in research facilities do not necessarily represent these field conditions adequately. They would however be best represented by assembling a challenge model that acknowledges this and uses a moderately pathogenic ETEC strain of the arguably highest prevalence in field (O149:F4).

Commercial piggeries are displaying huge differences depending on management, environmental conditions, unforeseeable incidents or human behavior. Inadequate cleaning and disinfection may allow pathogens to accumulate in the environment leading to increased infection pressure.

The standardized conditions in experimental facilities deviate from the conditions found in field. Hence, it has to be acknowledged that the established challenge model has a limited potential to be comparable to field conditions.

5.2.4 Establishment of a challenge model

Since there has been difficulty to reproduce PWD under experimental conditions (Chandler and Mynott, 1998) it had to be acknowledged that additional stressors beside the challenge with ETEC are needed. The mere challenge of healthy piglets with ETEC did not induce severe symptoms of disease (Opapeju, 2009), especially when weaning age is increased (Wellock et al., 2007). The severity is significantly promoted by adverse factors, that impair piglet health like early weaning age, low hygiene status, unfavorable climate conditions or unfavorable feed composition (Madec et al., 1998).

For reasons of practicability the whole trial had to be separated in three runs, each comprising 32 piglets. All piglets were handled and treated equally, however conditions varied, for instance due to individual or seasonal differences.

Piglets that took part in the three runs were controlled closely for signs of diarrhea during suckling period and scouring piglets were excluded from the experiment. Prior to infection all animals were controlled to be healthy with no signs of disease or discomfort. Three piglets were excluded from the trial.

Weaning age and acclimatization time of piglets before challenge were set in accordance with standards from prior piglet challenge trials (see table 1). It has to be taken into account that piglets of older age are better protected, because of an age dependent robust constitution that makes them less susceptible to diseases. The common practice of modern commercial piggeries for weaning age was used as orientation in the own trial. Duration of each trial period was three weeks, allowing for two weeks of clinical examination and observation after challenge. This time period does not allow for long-term investigations, but since severe health impairment and diarrhea are usually limited to a maximum of 3-5 days after challenge (Owusu-Asiedu et al., 2003; Rossi et al., 2012), this period was considered appropriate. Confirming this, no impact of challenge was noticeable on growth and feed intake from day 4 to 18 after challenge in another study (Trevisi et al., 2009).

The need for additional stressors influencing the occurrence of PWD has been discussed in the literature (Madec et al., 2000; Laine et al., 2008). In this study it was desisted to administer stress hormones prior to infection in order to generate limited stress levels in piglets. Bicarbonate for buffering of stomach acid to facilitate passage of challenge strain was also omitted, since this did not show clear results in previous experiments (Madec et al., 2000; Jamalludeen et al., 2009).

Piglets were given a short antibiotic treatment affecting the competing gut microbiota to facilitate adhesion of the infection strain and to allow for better comparability in the three runs. Starting on day of weaning until one day before infection, piglets were orally treated once daily with colistin sulphate (van Dijk et al., 2002; Niewold et al., 2007; Trevisi et al., 2009).

To test for presence of ETEC challenge strain the fecal cell numbers of the virulence factor genes *fae* (gene coding for the F4ac fimbrium) and *est-II* (gene coding for heat stable toxin Stb) were estimated by quantitative real-time PCR from fecal samples

rectally taken one day prior to infection. The *fae* gene was not detectable and *est-II* was detectable in only small numbers. It was therefore assumed that the ETEC challenge strain was not present in the population and that piglets could be considered immunologically naïve. This is in congruence with the insight that healthy animals can at times host and shed *E. coli* that carry virulence factors without showing clinical symptoms (Schierack et al., 2006b).

Because piglets were healthy and there were no hints or indices for disease, presence of other diarrheic pathogens (Transmissible Gastroenteritis; Epidemic viral diarrhea, Salmonellosis) was not differentially diagnosed.

Temperature in facilities was deliberately set at 23 ± 1°C, as the environmental temperature can promote PWD in piglets (van Dijk et al., 2002).

Feed is considered as an important factor influencing health and condition significantly. Increased crude protein content of the diet precipitated digestive problems and reduced fecal consistency in weaned piglets challenged with ETEC (Heo et al., 2009; Heo et al., 2010). A specific diet was formulated. In this study, crude protein content was increased compared to current recommendations, since decreased protein level reduced PWD and resulted in firmer feces (Wellock et al., 2007; Kim et al., 2011).

Piglets were challenged twice on day 3 post weaning in a 6 hours interval at an age of 25 days. The period between day 3-10 (Pluske et al., 2002) or 4–9 (Heo et al., 2009) respectively is regarded as the time of maximum colonization of the small intestine with ETEC. The gradual process of natural exposure to ETEC and subsequent colonization can hardly be simulated by artificial infection. It is however advisable to scatter infection dose to several points in time. The infection dose of 2 ml containing 5.0×10^9 cfu/ml was considered appropriate and corresponds to average values from other trials. For successful induction of PWD, the challenge dose seems to be of lower importance compared to the ability of the strain to colonize the gut and multiply (Bilkei, 1996; Stuke, 2003). Via the viable count of bacteria it was assured that bacteria were in the log phase of growth at the time of infection unfolding their maximal potential for infection. The F4ac fimbriae of the used challenge strain are most frequently found in PWD and are associated with low percentage of feces dry matter as well as high shedding of F4+ *E. coli* (Geenen et al., 2007). Knowing the F4 status of piglets should therefore be considered as

prerequisite for this type of trials (Niewold et al., 2007). In this study both parents of the piglets were confirmed to be F4 positive as described (Kreuzer et al., 2013) and only F4 positive piglets were used in this study. Failure to test or to choose piglets based on this characteristic has contributed to problems inducing PWD in earlier trials (Wellock et al., 2008).

For the challenge model it was crucial that symptoms observed could be attributed to ETEC colonization and the production of enterotoxins. However, piglets can be expected to show some variability. To provide more comparable microbiological and immunological conditions the use of specific pathogen free (SPF) or gnotobiotic animals might be an alternative.

Summarizing the results of this challenge model, a reproducible procedure was established , while the comparability to natural ETEC infections in the field seems to be questionable. This procedure could be repeated under identical conditions with similar results. It therefore appears justified to assume that the model would be useful for further studies of the interaction between ETEC and feed additives in piglets.

5.2.5 Effects of feed additives on the course of the disease

Various feed additives of different origin have previously been found to have a positive effect on piglet health. Based on these data a protective potential was ascribed to the investigated feed additives too.

It has to be stated that despite encouraging characteristics of used feed additives in the literature, no statistically significant effects on piglet health were measurable in the performed trials. However, the numbers of individual piglets per group was limited which has to be taken into account in this context.

This study observed a protective effect *in vitro,* while there was little to no protection of piglets in an experimental ETEC challenge trial. Similar results have been described in the literature (Harmsen et al., 2005).

Whether the dose of the feed additives, which was selected according to manufacturers' recommendations, was in the optimum range cannot be assessed. Dose finding trials were not subject of this study but would be necessary to explain effects or non-efficacy better.

Since there was a wide variety in acquired data it might appear reasonable to repeat studies using larger numbers of animals for any one group.

5.3   Conclusion

There is some evidence suggesting, that the yeast fermentation product could alleviate ETEC infection in piglets. This is warranted by statistically significant numbers *in vitro* and encouraging results *in vivo*, where the yeast fermentation product improved fecal characteristics. Protective properties of the feed additives Acid Mix and Thyme were not observed *in vitro*, but should be considered after results in challenge. Further research concerning the effects of the used feed additives appears necessary, including dose response studies, before recommendations can be given.

6. Summary

In vitro and *in vivo* studies on the effects of feed additives on a porcine intestinal epithelial cell line (IPEC-J2) and in weaned piglets experimentally challenged with enterotoxigenic *E. coli*

The intention of this study was to test both *in vitro* and *in vivo* whether feed additives have an inhibitory effect on *E. coli* infection of piglets.

The intestinal porcine epithelial cell line IPEC-J2 was used as *in vitro* model to assess effects of feed additives on adhesion of a F4 positive *E. coli* strain (ETEC). Feed additives were dissolved in cell medium, which was then used to grow cells in different feed additive concentrations. Cells were infected with a bacterial suspension previously stained with the fluorescent dye 5,6-carboxymethyl fluorescein diacetate succinimidyl ester. Bacterial adhesion was characterized by fluorescence intensity measured by flow cytometry of the IPEC-J2 cells.

A piglet feeding trial was performed in which 81 piglets were challenged with ETEC twice 72 and 76 hours after weaning. Piglets were fed a complete diet that met nutritional requirements and contained the feed additive in the concentration recommended by the producer. Health and performance of piglets were thoroughly examined during the whole course of the experiment (21 days). The objective of the challenge trial was to establish a suitable model for experimental infection (10^{10} cfu / animal) of weaner piglets (3 weeks old) using an ETEC strain positive for F4 fimbriae and both heat labile (LT1) and heat stabile (St1p and St2) toxins.

The *in vitro* trials showed that the yeast fermentation product has the potential to inhibit ETEC adhesion to IPEC-J2 cells. The relative fluorescence intensity of infected cells was reduced by 47.3 and 43.5% in the 10^{-2} and 10^{-3} % dilutions, the number of infected cells decreased by 94.7 and 80.4%, respectively.

The challenge trial resulted in marked clinical signs in all infected piglets and an overall loss of 16%. A marked decrease in fecal dry matter was observed in 83% of infected piglets.

Shedding of ETEC virulence factor genes *fae* and *est-II* was observed in 92.9% of the examined animals in high amounts. The tested feed additives were unable to

protect piglets after ETEC challenge. However, the feed additives Acid Mix and Thyme were able to produce results similar to those of the non-infected control group in feed intake and body weight.

Given the present data an influence of the investigated feed additives on intestinal physiology appears probable. However, to assess specific antibacterial potential further studies are required. Factors that potentially have a negative influence on effectiveness of feed additives (inclusion rate/dose in diet, effect of stomach pH, interactions with other feed components) should thereby be given particular attention.

7. Zusammenfassung

In-vitro- und *in-vivo*-Untersuchungen zum Einfluss von Futterzusatz-
stoffen auf eine intestinale Epithelzellkultur (IPEC-J2) sowie auf
Absetzferkel nach experimenteller Infektion mit enterotoxischen
E. coli.

Das Ziel dieser Studie war es, *in-vitro* und *in-vivo* zu testen, ob
Futtermittelzusatzstoffe eine hemmende Wirkung auf *E. coli*-Infektionen bei Ferkeln
haben.

Mögliche Effekte der Futterzusatzstoffe auf die Adhäsionsfähigkeit (erster Schritt der
Pathogenese) eines F4-positiven ETEC Stamms wurden zunächst *in-vitro* mit Hilfe
einer IPEC-J2 Zelllinie untersucht. Die Futterzusatzstoffe wurden dazu (soweit
wasserlöslich) in Zellmedium aufgelöst und dieses dann zur Anzucht der Zellen in
verschiedenen Konzentrationen der Futterzusatzstoffe verwendet. Die Zellkultur
wurde mit einer Bakteriensuspension infiziert, welche zuvor mit dem fluoreszierenden
Farbstoff CFDA versetzt worden war. Durch Messung der Fluoreszenzintensität der
IPEC-J2 Zellen mittels Durchflusszytometrie konnte anschließend die bakterielle
Adhäsionsfähigkeit beurteilt werden.

Weiterhin wurden in einem Fütterungsversuch 81 Ferkel aufgeteilt auf drei
Versuchsdurchgänge einer zweimaligen oralen Infektion mit ETEC 72 und 76
Stunden nach dem Absetzen unterzogen. Alle Ferkel erhielten dabei ein
bedarfsdeckend zusammengestelltes Alleinfutter, dem der Futterzusatzstoff in der
vom Hersteller empfohlenen Konzentration beigemischt war. Die Untersuchung der
Ferkel über die gesamte Dauer des Versuchs (21 Tage) beinhaltete die Erfassung
von Gesundheits- sowie Leistungsparametern. Weiteres Ziel des Versuches war es,
ein verlässliches Modell zur experimentellen Infektion (10^{10} cfu / Tier) von
Absetzferkeln mit F4-positiven, toxinbildenden (LT1, St1p, St2) ETEC zu etablieren.

Durch die *in-vitro*-Untersuchungen konnte eine verminderte bakterielle
Adhäsionsfähigkeit durch Einsatz des Hefepräparats PV beobachtet werden. In den
0,01- und 0,001-prozentigen Lösungen kam es hier zu einer Reduzierung der

Fluoreszenzintensität um 47 bzw. 43% sowie zu einer Verringerung des Anteils infizierter Zellen um 94 bzw. 80%.

Die Infektionsversuche führten bei allen Ferkeln zu deutlichen klinischen Symptomen bei einer Verlustrate von 16%. Bei 83% der infizierten Tiere kam es zu einer deutlichen Verringerung der Kottrockenmasse. Dabei schieden 92,9% der untersuchten Tiere den Erregerstamm nach der Infektion nachweisbar aus.

Durch die getesteten Futterzusatzstoffe konnten die Tiere zwar nicht statistisch relevant vor der ETEC Infektion geschützt werden. Allerdings erzielten Ferkel, denen das Additiv auf Basis von Thymianöl und das Gemisch organischer Säuren gefüttert wurde, zum Ende des Versuchs in den Leistungsparametern Futteraufnahme und Körpermasse ähnliche Werte, wie die Tiere der nicht-infizierten Kontrollgruppe.

Ein Einfluss der untersuchten Futterzusatzstoffe auf die Darmphysiologie erscheint auf Grund der vorliegenden Ergebnisse wahrscheinlich, zur genaueren Einschätzung des protektiven Potentials bedarf es jedoch weiterer Abklärung. Dabei sollten Faktoren, die die Effektivität des Futterzusatzstoffs vermindern können (effektive Dosis in der Diät, Magen pH-Wert, Interaktionen mit anderen Futterkomponenten) besondere Berücksichtigung finden.

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List of Publications

1. **Effect of different feed ingredients and additives on IPEC-J2 cells challenged with an enterotoxigenic Escherichia coli strain**

   Cytotechnology
   (Incorporating Methods in Cell Science - International Journal of Cell Culture and Biotechnology)

   Publication date: 15th of August, 2015

2. **A standardised challenge model with an enterotoxigenic F4+ Escherichia coli strain in piglets assessing clinical traits and faecal shedding of fae and est-II toxin genes**

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Declaration

I hereby declare, that the work presented is the original work of the author except as acknowledged in the text.

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Franz Spitzer