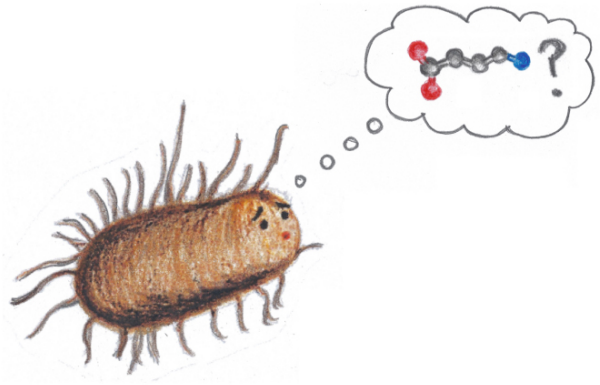
Aus dem Institut für Veterinär-Physiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

GABA, GadX and Gut Health:

How Stimulation of EPEC Transcription Factor GadX Can Improve the Gut Mucosal Barrier



Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der Freien Universität Berlin

vorgelegt von
Hannah-Sophie Braun
Tierärztin aus Berlin

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Contents

List of Abbreviations

AE lesions	attaching and effacing lesions
ABAT	4-aminobutyrate aminotransferase
CLA	conjugated linoleic acids
CNS	central nervous system
EAF	EPEC adherence factor plasmid
E. coli	*Escherichia coli*
EHEC	enterohemorrhagic *Escherichia coli*
ENS	enteric nervous system
EPEC	enteropathogenic *Escherichia coli*
Esp	EPEC-secreted proteins
ETEC	enterotoxigenic *Escherichia coli*
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
IPAN	intrinsic primary afferent neurons
L. rhamnosus	*Lactobacillus rhamnosus*
LEE	locus of enterocyte effacement
ler	locus of enterocyte effacement-encoded regulator
MUC1	mucin-1
NANC	nonadrenergic noncholinergic neurons
NO	nitric oxide
PER	plasmid-encoded regulator
PWD	postweaning diarrhoea

PER	plasmid-encoded regulator
PWD	postweaning diarrhoea
RpoN	alternative sigma factor 54
T3SS	type III secretion system
Tir	translocated intimin receptor
VIP	vasoactive intestinal polypeptide

List of Figures

1. General Introduction

Diarrhoeic diseases are among the most common health issues in humans and animals. Even though increasing hygienic standards and vaccinations have been able to decrease their prevalence significantly, they still present a world-wide health threat for both mankind and the animal kingdom (Lozano, Naghavi et al. 2012, Murray, Vos et al. 2012, Zambrano, Levy et al. 2014). Besides viruses, bacteria are the most frequent cause for the development of severe diarrhoea. However, their increasing resistance to antibiotics significantly impedes the treatment of bacteria-caused diseases - a threat so severe that it was discussed during the United Nations General Assembly High Level Meeting in 2016 leading to several political declarations world-wide.

Like many other enteropathogens, enteropathogenic *Escherichia coli* (EPEC) usually spread under poor hygienic conditions leading to severe illness in both humans and animals. In fact, endemic outbreaks of EPEC infections are a frequent cause of infant diarrhoea in developing countries (Ochoa and Contreras 2011). Moreover, EPEC appear to have the highest mortality rate of all bacterial pathogens causing diarrhoea in children less than 5 years of age (Lanata, Fischer-Walker et al. 2013).

Enteropathogenic *Escherichia coli* are common zoonotic pathogens in domestic animals such as pigeons, chicken, dogs or cats (Morato, Leomil et al. 2009, Gargiulo, Russo et al. 2014, Vasco, Graham et al. 2016). Also pigs are known to be a host of EPEC (Vasco, Graham et al. 2016). It is believed that the stocking rate and the resulting pathogen load generally influence the risk of infections in animal husbandry. In pig fattening, high stocking densities are commonly linked to an increased incidence of postweaning diseases as for example postweaning diarrhoea (PWD) (Buddle, Mercy et al. 1997, Skirrow, Buddle et al. 1997, Laine, Lyytikainen et al. 2008). Infections with EPEC are believed to be substantially involved in PWD, a multifactorial disease starting around 3-10 days after weaning (Vidotto, Florian et al. 2013). During this period, piglets are exceedingly susceptible to gut infections as they are affected by environmental and psychological stress, as well as by nutritional changes. Postweaning diarrhoea leads to massive economic losses due to higher mortality, decreased growth performance and reduced weight gain (Hampson, Woodward et al. 1993, Fairbrother, Nadeau et al. 2005, Laine, Lyytikainen et al. 2008).

For many years, it has been common practice in animal production to use antibiotics as feed additives, not only in order to prevent diseases but also to increase growth performance in general. However, the extensive use of antibiotics has led to an increased antibiotic resistance among various bacteria (Heo, Opapeju et al. 2013). Driven by growing concerns regarding the impact of antibiotic resistance on human health, the European Union has banned the use of antibiotics as feed additives in 2006. However, the actual demand for products treating bacterially caused diseases has further increased because the market for food of animal origin has continuously grown over the past decades (Hovhannisyan and Grigoryan 2016). In addition, since diarrhoeic diseases are one of the main factors influencing profitability of animal production (Fairbrother, Nadeau et al. 2005, Windeyer, Leslie et al. 2014, Mbanga and Nyararai 2015), the use of antibiotics appears even more tempting.

It can be expected that the spread of antibiotic resistance will continue. As a result, the ability to fight bacteria-induced diarrhoea effectively with antibiotics will be - at least in animal husbandry - impacted massively in the coming years. Moreover, political restrictions will lead to banning of certain drugs in animal farming, further reducing available options for antibiotic treatments. Therefore, a tremendous demand for alternative treatments and/or prevention methods for intestinal and other bacterial infections will emerge.

Many diarrhoeic diseases are induced multifactorially (Cox, Schrauwen et al. 1991, Gulliksen, Jor et al. 2009). Onset and course mostly depend on the interaction of several pathogens, the environmental conditions and the immunological response of the host. In fact, the severity of infection often depends on the host's condition, e.g. its immune status and the resulting interplay between host, pathogen and environment. These interdependencies offer a tremendous potential for the development of novel, more effective approaches to prevent and treat bacterial infections by modulating both, the bacterial attack and the response of the host. The development of such prevention strategies requires further research on the bacterium-host interaction. In terms of enteric infections, two approaches seem to be promising: the repression of virulence factors of potential pathogens and the enhancement of gastrointestinal defence mechanisms of the host which reduces its intestinal susceptibility to infections.

In case of EPEC infections, the regulation of virulence genes is strongly linked to the production of the non-proteinogenic amino acid γ-aminobutyric acid (GABA). Since GABA is also known as a neurotransmitter with various effects on the host, the interaction of host and bacterium via GABA as a signal molecule will be the central topic in this work.

2. Literature Review

2.1. General Overview of GABA

Gamma-aminobutyric acid (GABA) is known as a major inhibitory transmitter of the central nervous system (CNS) (Watanabe, Maemura et al. 2002). It is present in nearly one third of the human neurons (Purves, Augustine et al. 2001) but is also produced by various non-neural tissues in humans and animals and can even be found in plants and bacteria (Obata 2013).

GABA is a non-proteinogenic amino acid. Conformation of GABA strongly depends on the environment, but under physiological conditions it usually appears as a zwitterion with a deprotonated carbon group and a protonated amino group, which impedes crossing the blood-brain barrier (Roberts and Sherman 1993).

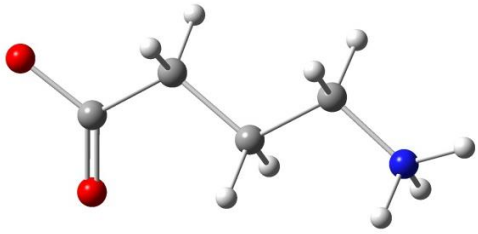

Figure 1: Molecular Structure of GABA

= Carbon

= Oxygen

= Nitrogen

= Hydrogen

The inhibitory function of GABA on the mammalian CNS is carried out by binding to different types of pre- or postsynaptic GABA receptors, thereby causing a hyperpolarization or - depending on the cell type - a depolarization of the cell (Wang, Summers et al. 2015). GABA receptors are widely distributed within the body and can even be found in the intestine (Erdo and Wolff 1990, Watanabe, Maemura et al. 2002). The synthesis of GABA in neurons is dependent on the enzyme glutamic acid decarboxylase (GAD) with its two isoforms GAD 65 and 67. GAD catalyses the decarboxylation of glutamic acid into GABA (Tillakaratne, Medina-Kauwe et al. 1995). As is the case with GABA, GAD can not only be found in the CNS but also in the enteric nervous system (ENS) as for example in the ileum of mice, rats and guinea pigs (Williamson, Faulkner-Jones et al. 1995).

Even though GABA was discovered in the early 20th century (Bowery and Smart 2006), it took decades until the relevance of this relatively simple molecule was recognized as one of the major neurotransmitters of the CNS: in 1950, GABA was identified in the mouse brain by Robert and Frankel (Roberts and Frankel 1950) and Awapara et al. (Awapara, Landua et al.

1950). Only 15 years later, Curtis and Watkins revealed the ability of GABA to inhibit action potentials (Curtis and Watkins 1965).

Before the relevance of GABA as principal inhibitory transmitter of the CNS was discovered, it was long known as a linking metabolite of carbon and nitrogen metabolism of plants (Michaeli and Fromm 2015). Besides being a metabolite, the meaning of GABA in plants is not fully elucidated yet, but it is thought to serve as signalling molecule (Bouche, Lacombe et al. 2003) and to be involved in the stress response (Bouche and Fromm 2004, Michaeli and Fromm 2015). The pathway of GABA production seems to be highly conserved in all species since the synthesis of bacterial, mammalian and plant-derived GABA is - at least in parts - similar (Chung, Bown et al. 1992, Takayama and Ezura 2015). Usually the GABA concentration in plant cells ranges between 0.03 to 2.00 µmol g^{-1} fresh weight (Rhodes, Handa et al. 1986, Fougere, Le Rudulier et al. 1991, Shelp, Bown et al. 1999) but can increase up to 40 times due to heat shock, hypoxia or other forms of stress response but also due to mechanical stimulation (Shelp, Bown et al. 1999, Petrivalsky, Brauner et al. 2007). As in bacteria (Chapter 2.3), the GABA pathway of plants might also contribute to the stabilization of the cytosolic pH by eliminating protons from the cytosol (Rolin, Baldet et al. 2000).
The hyperpolarizing effect of GABA as inhibitory transmitter is not only limited to vertebrates but also occurs in invertebrate animals. This can be beneficial for plants: e.g. phytophagous activity of insects leads to a destruction of plant cell structures which results in a stimulation of GABA synthesis and a subsequent accumulation of GABA in the affected tissue. As a consequence, the vermin takes up high amounts of GABA which are believed to inhibit its normal growth and development (Ramputh and Bown 1996).

Interestingly, it has also been shown that GABA acts as signalling molecule between plants and bacteria: e.g. tomatoes raise their GABA production in wounded tissues to increase the extracellular GABA concentration. The excess of GABA leads to a reduced virulence of certain attaching bacteria (Chevrot, Rosen et al. 2006, Shelp, Bown et al. 2006).

Due to the fact that GABA seems to have crucial functions in plants resulting in different quantities of GABA in all tissues, it is not surprising that food and feed can contain considerable amounts of GABA. Own experiments presented in Chapter 4 revealed that GABA concentrations in pig feed range around 180 µmol/l. There is also a variety of GABA-rich food available such as rice or tea, claiming natural tranquillization and reduction of anxiety. However, a direct impact of orally administered GABA on the CNS has not been proven so far since GABA is not able to cross the blood-brain barrier. Instead, an effect of food-derived

GABA on the gut seems likely. Furthermore, abdominal vagal afferents were found to be involved in emotional behaviour, indicating a potential role of GABA on the gut-brain axis and "gut feelings" (Klarer, Arnold et al. 2014). The influence of GABA on the gut and the brain will be described in Chapter 2.4.3.

2.1.1. GABA Metabolism

The synthesis of GABA is carried out by different enzymes. The most common pathway is the decarboxylation of L-glutamic acid by GAD, which is highly conserved in plants, bacteria and mammals. In mammals, two isoforms of GAD are known: GAD 65 and GAD67 with the respective molecular weights of 65,000 Da (GAD65) and 67,000 Da (GAD67) (Tillakaratne, Medina-Kauwe et al. 1995). Both isoforms are believed to be derived from a common ancestral GAD gene that is conserved throughout various species (Bu and Tobin 1994). While GAD65 and GAD67 show a high homology among different species, the similarity between both isoforms within a species is rather low. For example, it was demonstrated that the rat GAD isoforms only show 63% of sequence similarity (Bu, Erlander et al. 1992). Usually expression of one isoform dominates, depending not only on the tissue but also on the interneuronal distribution (Feldblum, Erlander et al. 1993, Fish, Sweet et al. 2011). Due to the fact that GABA metabolism in the brain is believed to be involved in the development of several neurological and neuropsychiatric disorders (Reichel, Nissel et al. 2014), GAD expression has been widely studied in neuronal tissues. Today it is well-known that GAD67 is present throughout all parts of the GABA-ergic neurons, whereas GAD65 can predominantly be found in axon terminals (Esclapez, Tillakaratne et al. 1994). The reason for the existence of both isoforms in one cell type is still not clear.

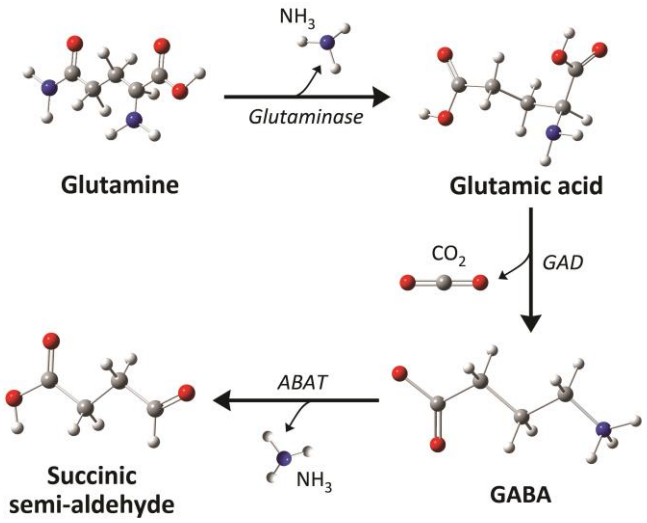

Figure 2: **GABA Metabolism**

= Carbon

= Oxygen

= Nitrogen

= Hydrogen

Only a few studies addressed the evaluation of GAD65 and GAD67 levels in non-neural tissues, but it was shown that both isoforms are expressed in various cell types such as endothelial, epithelial, endocrine and germ cells (Erdo 1992). In the present study (Chapter 4), an – albeit weak – mRNA expression of GAD65 but not GAD67 was found in porcine intestinal epithelium.

The substrate of GAD, glutamic acid, is mainly converted from glutamine. The reaction is performed by the enzyme glutaminase, catalysing a deamination (Figure 2). Besides the above-mentioned pathway, GABA can also be derived via deamination and decarboxylation from putrescine, spermin, spermidine and ornithine (Seiler 1980, Tillakaratne, Medina-Kauwe et al. 1995).

The enzyme 4-aminobutyrate aminotransferase (ABAT), also called GABA transaminase, initiates the breakdown of GABA by catalysing the reaction of GABA to succinic semi-aldehyde, which is then further converted to succinate (Michaeli and Fromm 2015). Succinate can enter the tricarboxylic acid cycle. Several ABAT inhibitors are used as antiepileptic drugs. By decreasing the GABA breakdown rate, they lead to enhanced GABA levels in the CNS (Angehagen, Ben-Menachem et al. 2003).

Bacterial GABA metabolism is described in Chapter 2.3.

2.1.2. GABA-Receptors

Depending on classification, GABA receptors can be divided into two or three different receptor types which are widely distributed within the body. They can be found predominantly in the CNS but also in the intestine, stomach, pancreas, uterus, ovary, testis, kidney, urinary bladder, lung, and liver (Erdo and Wolff 1990)**.**

Previously, GABA receptors have been divided into two types: ionotropic and metabotropic (G-protein coupled) receptors. Nowadays, GABA receptors are typically classified as $GABA_A$ and $GABA_C$, both ionotropic receptors, and $GABA_B$, a metabotropic receptor (Blein, Hawrot et al. 2000).

$GABA_A$ and $GABA_C$ receptors are pentameric proteins composed of several distinct polypeptides which form four transmembrane domains. $GABA_A$ receptors consist of α, β, γ, δ, ε, θ, π and ρ subunits (Olsen and Tobin 1990, Mehta and Ticku 1999) whereas $GABA_C$ receptors only consist of ρ subunits. Both receptors are ligand operated ion channels with a binding site for GABA.

The inhibitory effect of receptors $GABA_A$ and $GABA_C$ is carried out by shifting the membrane potential to highly negative values that impede the signal transmission of an incoming action potential. The ion channel of both receptor types is activated by binding of two molecules of GABA, leading to an influx of chloride ions (Cl^-). The influx of negatively charged Cl^- causes a rapid hyperpolarization of the postsynaptic membrane, inhibiting the firing of action potentials (Smith and Olsen 1995).

The $GABA_B$ receptor is a metabotropic transmembrane receptor consisting of heterodimeric combinations of two isoforms ($GABA_B1$ and $GABA_B2$) depending on its localization (pre- or postsynaptic) (Froestl 2011). Unlike $GABA_A$ and $GABA_C$ receptors, $GABA_B$ receptors provoke a slow but rather prolonged neuronal inhibition and activation requires a stronger or longer stimulation (Bowery 1989, Sodickson and Bean 1996). This effect is mediated by linking of the $GABA_B$ receptor to the heterotrimeric guanine-nucleotide binding (G) proteins, G_i and G_o. Hence, the receptor indirectly modulates the activity of the adenylyl cyclase, resulting in either an activation of inwardly rectifying potassium channels (the term inwardly rectifying is used despite the fact the receptor activation induces an outward flow of potassium ions after activation) or a suppression of voltage dependent calcium (Ca^{2+}) channels. In the postsynaptic membrane, binding of GABA causes an increased permeability of potassium channels and a subsequent efflux of potassium ions (K^+). As a consequence, the postsynaptic membrane hyperpolarizes, reducing the chance of the occurrence of successful action potentials (Bowery 1989, Kerr and Ong 1995, Bettler, Kaupmann et al. 1998).

In the presynaptic neuron, stimulation of $GABA_B$ receptors activates potassium channels but also leads to a suppression of Ca^{2+} channels. The subsequent reduction of Ca^{2+} concentration in the cell inhibits either the GABA release from GABA-ergic nerve terminals or the release of other neurotransmitters, e.g. glutamate, from the respective nerve terminal (Chalifoux and Carter 2011).

2.1.3. Function of GABA

As described above, GABA is an inhibitory transmitter of the CNS. By binding to GABA receptors, the subsequent ion flow (either an influx of Cl^- or an efflux of K^+, depending on the receptor) causes a hyperpolarization of the respective neuron which leads to sedative, antiepileptic, pain killing or muscle relaxing effects (Carai, Colombo et al. 2001). Strikingly, the GABA precursor glutamate is the direct counterpart of GABA, acting as the most abundant excitatory neurotransmitter of the brain (Meldrum 2000).

Typical effects of GABA such as muscle relaxation and pain reduction are well investigated and can be comprehensively explained by the hyperpolarization of the respective neurons. Less coherent is the role of GABA in the complex system of mood and behaviour. It has been shown that increased GABA levels are responsible for the postpartum suppression of anxiety-related behaviour (Lonstein, Maguire et al. 2014). Furthermore, GABA seems to play a central role in patients with anxiety disorders and depression (Mohler 2012). Due to the significance of the GABAergic system for important physiological functions and the devastating consequences when the system is disturbed, many different approaches have been developed that target at GABAergic neurons. GABA reuptake inhibitors like tiagabine, which block GABA transporters and lead to a subsequent increase of extracellular GABA, are evaluated for their potential to treat anxiety disorders (Pollack, Roy-Byrne et al. 2005). Pregabalin, a GABA analogue, is commonly used for the treatment of neuropathic pain, epilepsy or anxiety disorders (Schifano 2014). Also the previously mentioned $GABA_A$ receptor agonists such as benzodiazepines are frequently used in the treatment of psychiatric disorders.

Various further aspects of the function of GABA in the CNS may be worth mentioning here. However, central effects of GABA are beyond the scope of this work. Therefore, the following text will primarily illuminate the role of GABA outside the CNS, particularly in the gut.

2.1.3.1. Effects of GABA in Non-Neural Tissues

Besides the typical function as an inhibitory transmitter in the CNS, there is evidence that GABA also plays a role in non-neural tissues. For many years, it was believed that GABA receptors can only be fully activated by millimolar levels of GABA. Hence, findings proving that GABA levels outside the CNS are much lower were taken as evidence against the existence of a peripheral GABAergic system. When it was revealed that very low concentrations of GABA (nanomolar to micromolar range) are sufficient to activate GABA receptors (Birnir, Everitt et al. 1994, Lindquist and Birnir 2006, Jin, Jin et al. 2011, Jin, Mendu et al. 2013), the significance of GABA for tissues outside the CNS became highly obvious.

Indeed, several endocrine cells outside the mammalian brain are known to contain GABA. However, the existence of GABA or its related enzymes in endocrine cell types such as adrenal chromaffin cells or pinealocytes seems coherent as those cells are derived from neuroepithelial cells and might, therefore, retain some neural properties. Surprisingly, some endocrine tissues of non-neural origin – e.g. in the stomach – have also been demonstrated to contain GABA (Erdo and Wolff 1990), implying the existence of a peripheral GABA system. Furthermore, GABA, ABAT and GAD were found to be produced in the pancreatic β -cells of rats (Vincent,

Hokfelt et al. 1983, Garry, Sorenson et al. 1987), in which GABA is believed to play a role in the regulation of β-cell function and the stimulation of insulin secretion.

As it was shown in the present study (Chapter 4), also epithelial cell types- such as intestinal epithelia- were found to contain some constituents of a GABA system. Cells of the proximal tubules of the kidney seem to produce GABA (Goodyer, Mills et al. 1982) and even in hepatocytes, GABA and GABA receptors have been detected (Minuk 1993). Both GAD 65/67 and GABA were also found in the rat oviduct. Interestingly, immunohistochemistry revealed that more GABA and GAD 65/67 were present in the mucosal epithelium compared to neurons, strongly indicating a non-neural origin (Erdo, Joo et al. 1989, Tillakaratne, Erlander et al. 1992). In the same study, GAD 67 was found in rat testis, specifically in spermatocytes. The function of GABA and GAD in the oviduct or the spermatocytes is still a matter of debate, ranging from morphogenetic to endocrine or even energy sourcing functions (Erdo and Wolff 1990, Persson, Pelto-Huikko et al. 1990, Ritta, Campos et al. 1991).

Expression of both GABA receptors and GABA-related enzymes has been detected in airway epithelium (Xiang, Wang et al. 2007, Mizuta, Osawa et al. 2008). In addition, GAD expression was found to be positively correlated with mucin expression and the hyperplasia of goblet cells. Accordingly, GABA antagonists repressed mucin expression in cultured pulmonary epithelial cells (Xiang, Wang et al. 2007, Wang, Wang et al. 2010). The own studies presented in this thesis elucidated that a correlation of GABA and mucin expression exists also in jejunal epithelium of pigs (Chapter 4). Furthermore, mRNA expression of $GABA_B$ receptors was detected in the porcine intestinal mucosa; whereas, Li et al. found evidence for the existence of $GABA_A$ receptors in the gut epithelium of pigs, mice and humans (Li, Xiang et al. 2012). Further described effects of GABA on the intestines range from decreased to increased motility. It has been discussed that those effects may be caused by inhibitory effects on intramural cholinergic neurons or a direct activation of the intestinal smooth muscles (Auteri, Zizzo et al. 2014). Nevertheless, a reduced gastrointestinal motility appears to be the predominant effect of GABA application in the gut.

In addition, GABA is discussed to influence immunological functions. In the gut, mucins are within the first line of immunological response to bacterial invasion, affecting the colonization by various enteric bacteria. In the presented study (Chapter 4) it was demonstrated that GABA is involved in the regulation of intestinal mucin expression which makes GABA part of the complex regulatory system of the gut´s immune response. Additionally, GABA was observed to enhance immunological reactions under stress conditions (Abdou, Higashiguchi et al. 2006,

Jin, Mendu et al. 2013). Furthermore, it has been demonstrated that GABA mediates the inhibition of T cell proliferation through $GABA_A$ receptors which potentially attenuates immune responses (Tian, Chau et al. 1999). In a study by Jin et al., the intravenous administration of GABA in rats led to a significant increase of intestinal IgG and IgA secretion (Jin, Guo et al. 1989). In growing chicken, the immunological response to heat stress was enhanced after supplementing the feed with GABA (50mg/kg body weight). Plasma levels of interleukin-2, IgA, IgG, and IgM and the levels of B and T lymphocytes were found to be significantly increased, indicating an improved development of immune functions (Tang and Chen 2015).

2.1.3.2. Intestinal Effects of GABA

During the past decades, the impact of GABA on gastrointestinal functions such as motility, secretion or immune response has been widely discussed. Nowadays, it is believed that these effects are not only mediated by the role of GABA as a neurotransmitter of enteric interneurons - even though this role might be the most obvious one - but also via pathways without direct involvement of enteric neurons. However, the specific role of GABA within the ENS is not clear yet: GABA, GAD or GABA receptors were found in enteric neurons of the submucosal and myenteric plexus and mucosal endocrine-like cells (Auteri, Zizzo et al. 2014), indicating an involvement in both motoric and secretory functions of the gastrointestinal tract. Since no evidence was found for a response of nerve-free gastrointestinal smooth muscles upon GABA administration - although smooth muscles have been reported to express GABA receptors - the influence of GABA on gut motility is most certainly induced through myenteric neurons. The latter were frequently shown to contain GABA (Grider and Makhlouf 1992). In the large intestine, GABAergic neurons account for 5 to 8% of the total myenteric neurons (Hyland and Cryan 2010).

It has been shown that secretory effects of GABA are also mediated by direct action on non-neuronal tissues such as the gut epithelium (Li, Xiang et al. 2012). In addition, there is increasing evidence that GABA induces other effects without direct neuronal involvement, in particular on the gut´s immune system and on the intestinal microbiota. The influence of GABA on gut bacteria and vice versa will be elucidated in detail in the next chapters.

As mentioned previously, one of the most obvious effects of GABA on the gut is probably its impact on gastrointestinal motility, primarily causing a reduced activity of enteric smooth muscles. However, contrary to the typical hyperpolarization which is usually induced by binding of GABA to its receptors, intestinal GABA cannot only lead to an inhibitory but also

to a stimulating effect. Depending on the species, the respective GABA receptors and the gastrointestinal segment involved, GABA was found to induce either relaxation or contraction of gastrointestinal smooth muscles (Auteri, Zizzo et al. 2014, Kaewsaro, Nualplub et al. 2017). The muscular response to $GABA_A$ and $GABA_C$ receptor activation is usually carried out by a neurotransmitter release from cholinergic and nonadrenergic noncholinergic (NANC) neurons. Surprisingly, $GABA_A$ and $GABA_C$ receptor activation can lead to either contraction or relaxation depending on the intracellular Cl^- level of the respective enteric neuron. It is believed that the Cl^- influx following the activation of a $GABA_A$ receptor could produce a depolarization of the membrane if the cell already shows a high intracellular Cl^- level (e.g. caused by an upregulation of the Na^+-K^+-$2Cl^-$ symporter). This effect was formerly reported in studies concerning brain development, which indicated that $GABA_A$ receptor activation does not only lead to the typical hyperpolarization of the cell membrane but can also induce an excitatory response (Auteri, Zizzo et al. 2014, Watanabe and Fukuda 2015). Other studies revealed that the activation of $GABA_B$ receptors reduced acetylcholine release from enteric neurons resulting in the typical inhibitory effect (Cherubini and North 1984, Frigo, Galli et al. 1987, Krantis and Harding 1987, Zizzo, Mule et al. 2007), which leads to a decreased motility of the intestine.

Because partly differing effects of GABA have been observed in different parts of the gastrointestinal tract, the segment-specific effects shall be analysed in more detail in the following subsections.

Stomach: It is well described that GABA receptor signalling is significantly contributing to maintaining the balance of gastric motor and secretory functions. However, the regulation processes are very complex and still far from being well understood.
Most of the studies evaluating the effect of GABA administration to the stomach report excitatory effects of GABA. It is believed that such effects are predominately induced by $GABA_B$ receptors, which either stimulate acetylcholine release from cholinergic neurons of vagal afferents or reduce the release of vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) from NANC neurons (Auteri, Zizzo et al. 2014). Due to its clinical relevance related to the treatment of gastroesophageal reflux disease, the $GABA_B$ receptor and its influence on motility and secretion of the stomach has been intensely studied. For example, in mice and ferrets, $GABA_B$ receptor activation increased gastric tone, contraction amplitude and also gastric acid release (Andrews and Wood 1986, Andrews, Bingham et al. 1987, Piqueras and Martinez 2004). An activation of $GABA_A$ receptors, in turn, is believed to mediate gastric relaxation. In a study by Rotondo et al., GABA, the $GABA_A$ receptor agonist muscimol and the

$GABA_B$ receptor antagonist phaclofen were reported to induce gastric relaxation in mice, indicating that $GABA_A$ receptors might stimulate the release of NO from intrinsic neurons (Rotondo, Serio et al. 2010). In rats, similar results were obtained (Krantis, Mattar et al. 1998). In contrast, in a study by Tsai et al. contradictory responses were observed in the guinea pig stomach: both GABA and muscimol administration, caused a contraction of the longitudinal muscle strips, which was inhibited by bicuculline, a selective $GABA_A$ receptor antagonist (Tsai, Tsai et al. 1993).

The variable results of different studies can be partly explained by the fact that effects of GABA can be induced both by central GABA receptors and by local GABA receptors in the gut. Some authors suggest that the interplay between local and central effects is important for the fine tuning of the gastrointestinal functions (Gyires, Toth et al. 2015, McMenamin, Travagli et al. 2016) .

Duodenum: In general, $GABA_A$ and $GABA_B$ receptor effects in the small intestine seem to be contrary to those observed in the stomach. $GABA_A$ receptor activation is rather linked to excitatory effects, resulting in muscle contractions, whereas $GABA_B$ receptor activation induces relaxation (Auteri, Zizzo et al. 2015).

In the duodenum, GABA was demonstrated to induce either a purine release (Maggi, Manzini et al. 1984) or a release of NO or VIP from NANC neurons (Krantis, Mattar et al. 1998), resulting in a duodenal relaxation, which was thought to be elicited by $GABA_A$ receptors. A subsequent study revealed that the observed effect of NO was mainly caused by $GABA_C$ receptor activation, whereas $GABA_A$ receptors acted on excitatory neurons, leading to an acetylcholine release (Zizzo, Mule et al. 2007). Not much is known about the role of $GABA_B$ receptors in the duodenal functions. However, own studies revealed the mRNA expression of $GABA_B$ receptors in the porcine duodenal epithelial and muscular layers, indicating a potential involvement in both motility and secretion (Chapter 4). Additionally, two older studies reported an inhibitory effect of baclofen, a $GABA_B$ receptor agonist, on electrically evoked duodenal contractions (Krantis and Harding 1987, Barbier, Guenaneche et al. 1989). Since $GABA_B$ receptor-expressing cells in the rat duodenum were frequently found to contain serotonin, some authors suggest a possible interplay of $GABA_B$ receptors and the serotonergic system (Nakajima, Tooyama et al. 1996, Auteri, Zizzo et al. 2015).

Jejunum: Described effects of GABA in the jejunum range from NANC-mediated muscle relaxation to contractions elicited by increased acetylcholine release of cholinergic neurons. As

in upper parts of the gastrointestinal tract, GABA´s divergent effects on motility are believed to be attributed to the response of different GABA receptor types.
In rat, the stimulation of $GABA_A$ receptors was shown to increase acetylcholine release, resulting in a muscle contraction. In contrast, $GABA_B$ receptor activation caused a decrease in electrically evoked contractions, implying a declined acetylcholine release (Krantis and Harding 1987). Conformingly, in a study performed in human jejunum, a dose-dependent decrease in spontaneous contractions of the longitudinal muscle was reported after $GABA_B$ receptor agonist administration. This was not observed after administration of $GABA_A$ receptor agonists (Gentilini, Franchi-Micheli et al. 1992). Considering that the gastrointestinal response to GABA obviously differs substantially between species, more studies are required to understand the complexity of the GABAergic system and its influence on the jejunal motility.

Ileum: The ileal response to GABA is as divergent as it is described for the more oral segments of the small intestine. $GABA_A$ receptor activation was found to mediate both excitatory and inhibitory effects. In several studies, GABA or $GABA_A$ receptor agonist-induced activation of cholinergic neurons simultaneously caused an elevated release of acetylcholine (resulting in an excitatory effect) and an increase in NO synthesis (resulting in an inhibitory effect/muscle relaxation) (Krantis, Costa et al. 1980, Giotti, Luzzi et al. 1983, Hebeiss and Kilbinger 1999, Kilbinger, Ginap et al. 1999). In contrast, $GABA_B$ receptors were found to mediate a solely inhibitory effect by decreasing acetylcholine release from cholinergic neurons, which reduced the amplitude of ileal contractions *in vitro* (Ong and Kerr 1982, Krantis and Harding 1987).
Conformingly, Pencheva et al. found a dose-dependent biphasic response of GABA administration on a longitudinal or circular muscle preparation of the cat´s ileum (Pencheva, Itzev et al. 1999): GABA caused a change of contraction and relaxation in spontaneous muscle activity in terminal and distal parts of the ileum. However, the pattern was different when GABA was applied to proximal parts of the ileum as only a monophasic response – a contraction – could be observed. Differences were also observed related to the muscle layer (longitudinal vs. circular). These findings can probably be attributed to different expression patterns of GABA receptor types within in ileum. Taken together, the strongly variable effects of the activation of particularly $GABA_A$ receptors as well as the findings about their locally varying expression suggest that GABA is a vital part of the fine modulation of the cholinergic signalling in the ileum (Auteri, Zizzo et al. 2015).

Large intestine: Most studies on the large intestines were carried out in the colon. Motility in the colon is mainly determined by the peristaltic effect. The colonic peristalsis is essentially

driven by the ENS, even though it is triggered by the parasympathetic nervous system, whereas the sympathetic nervous system has an inhibitory effect. Acetylcholine and substance P as well as NO, VIP and serotonin are involved in this complex process, which is characterized by an oral contraction and an aboral relaxation. The resulting peristaltic waves transport the digesta in an aboral direction (Grider 2003). It is not surprising, that GABA plays a role in the modulation of the motility of the large intestine, even though it was shown that GABA is not crucial for the colonic peristalsis (Frigo, Galli et al. 1987, Tonini, Crema et al. 1989).
Similar to the ileum, GABA was shown to induce either a monophasic relaxation or a biphasic response (relaxation followed by a contraction) in the rat colon, which was inhibited by bicuculline, a $GABA_A$ receptor antagonist, and mimicked by muscimol, a $GABA_A$ receptor agonist (Bayer, Crenner et al. 2002). These effects are probably caused by either a stimulation of neurotransmitter release from NANC neurons or an increase in acetylcholine release from cholinergic neurons (Auteri, Zizzo et al. 2014). In turn, the $GABA_B$ receptor agonist baclofen was shown to decrease acetylcholine release of cholinergic neurons, leading to a depression of the cholinergic tone of the smooth muscle in unstimulated longitudinal and circular muscle preparations of rabbit colon (Tonini, Crema et al. 1989). Even though the contractile response to transmural stimulation was not essentially depending on the influence of GABA, it was assumed that an intrinsic GABAergic pathway is involved in the fine-tuning of both the excitatory cholinergic and the inhibitory nonadrenergic noncholinergic response that modulates peristalsis.

2.2. Enteropathogenic *Escherichia coli*

EPEC are Gram-negative bacteria, which can mainly be found in the intestinal tract of endothermic organisms (Donnenberg 2013). The first EPEC strains were described in 1955, when a relation to severe diarrhoea outbreaks became evident (Neter, Westphal et al. 1955).

EPEC play a major role in infant diarrhoea in developing countries where EPEC infections still entail a significant risk for death (Kotloff, Nataro et al. 2013). This is mainly caused by the induction of secretory diarrhoea with significant losses of electrolytes and water, which leads to life-threatening exsiccation. However, it has also been shown that EPEC strains do not necessarily provoke severe symptoms as they have been isolated from healthy hosts, both human and animals, or hosts which show only weak symptoms (Alikhani, Mirsalehian et al. 2006).

It is believed that the variation in the severity of symptoms depends on the EPEC strain and its set of virulence genes, which is significantly influencing the adherence patterns. Many attempts have been made to identify the exact genetic causes responsible for *E. coli* pathogenicity. However, the variety of serotypes and genetic virulence patterns makes it difficult to distinguish pathogenic from rather non-pathogenic strains on a genetic level, in particular because many virulence genes are only expressed under certain environmental conditions.

The major virulence genes inducing the pathogenicity of EPEC are located on the locus of enterocyte effacement (LEE), a 36.5kB chromosomal pathogenicity island consisting of five different polycistronic subunits (LEE1-5), which was first described in 1995 by McDaniel et al. (McDaniel, Jarvis et al. 1995). LEE includes genes as the type III secretion system (T3SS), a specialized system to secrete effector proteins into the host cell. The T3SS genes are located on LEE1-3, encoded by the genes *sep* and *esc* (Elliott, Wainwright et al. 1998, Mellies, Elliott et al. 1999). Together with proteins required for the intimate attachment – e.g. intimin and the translocated intimin receptor (Tir) that are also encoded on LEE - these genes are mainly responsible for the formation of attaching and effacing lesions (AE lesions). Not only EPEC but also enterohemorrhagic *E. coli* (EHEC), *Citrobacterium rodens* and rabbit-specific EPEC are able to produce such AE lesions which lead to a massive destruction of the enteric brush border (Marches, Nougayrede et al. 2000, Mundy, MacDonald et al. 2005, Croxen and Finlay 2010, Bustamante, Villalba et al. 2011).

After entering the host´s gastrointestinal tract orally (mainly via infected food), the bacteria cluster non-intimately to the intestinal mucosa. Subsequently, a set of EPEC-secreted proteins (Esp), including Tir, is inserted via the T3SS into the epithelial cells from where it interacts with the outer membrane protein intimin. The interaction leads to an intracellular signalling pathway of the enterocyte, which has not been fully elucidated yet. It is believed that this signalling pathway induces a loss of microvilli and a rearrangement of the epithelial cell skeleton, which enables the bacterium to attach intimately to the enterocyte membrane. As a result, a destruction of the enteric brush border with the typical AE lesions occurs (Nougayrede, Fernandes et al. 2003, Hayward, Leong et al. 2006, Bustamante, Villalba et al. 2011).

Since the major virulence genes of EPEC are located on LEE, this gene locus has been in the spotlight for research over many years. However, expression of LEE genes underlies a complex regulation and is influenced by temperature, pH, growth phase and quorum sensing (Abe, Kenny et al. 1997, Kenny, Abe et al. 1997, Kaper and Sperandio 2005). Various genetic regulators encoded inside and outside of LEE have been described. One of them is the locus of

enterocyte effacement-encoded regulator, called *ler* which is a 15kDa protein harboured on the first gene of the LEE1 and acts as a critical positive regulator of the LEE (Franzin and Sircili 2015).

How *ler*-related LEE activation works in detail has not been fully elucidated; especially the fact that *ler* represses its own transcription on LEE while activating all other LEE genes is not easily interpreted (Haack, Robinson et al. 2003, Garcia, Cordeiro et al. 2012, Bhat, Shin et al. 2014). Expression of *ler* seems to counteract the HN-S protein, a global transcriptional regulator that can act as repressor of LEE. Hence, *ler* mutants are unable to secret proteins of the T3SS and to induce AE lesions (Elliott, Sperandio et al. 2000).

Regulation of *ler* expression itself depends on the genetic set up of the bacterium. In typical EPEC, the plasmid-encoded regulator (PER) plays an important role in *ler* regulation. The current classification of EPEC depends on the presence or absence of the EPEC adherence factor (EAF) plasmid. Strains harbouring the EAF belong to the group of typical EPEC, whereas strains lacking EAF are defined as atypical EPEC. Among others, the EAF contains two important gene loci: the locus of the bundle forming pilus (bfp), which is often used for the detailed characterization of the strain, and PER (Contreras, Ochoa et al. 2010).

In typical EPEC, PER is known to activate *ler.* Expression of PER, in turn, is induced by many factors like environmental conditions and quorum sensing. However, there are many more factors that lead to an activation of *ler*, which explains why also atypical EPEC express LEE genes although they are lacking the EAF plasmid and thereby PER. The regulation of virulence in EPEC is still far from being fully understood due to its complexity. The same growth conditions often lead to significantly different expression levels of virulence genes in different EPEC isolates, which make it difficult to define the regulatory processes that are responsible for the activation or repression of LEE gene expression.

Interestingly, the LEE is not only crucial for pathogenicity of EPEC but also for that of EHEC. Major pathways of pathogenesis and virulence gene expression, including the GABA-dependent acid-regulation system are similar in EPEC and EHEC (Nguyen and Sperandio 2012). However, EHEC and EPEC prefer different sites of attachment. EHEC has a distinct tropism for the epithelium of ileal Peyer`s patches whereas EPEC also attach in other parts of the small intestine such as the jejunum and in some species in the colon (Phillips, Navabpour et al. 2000).

Even though some findings and conclusions herein might also be transferable to EHEC, this work will focus mainly on EPEC in the small intestine.

2.3. Bacterial GABA Production

GABA is produced by EPEC and other enteric bacteria in order to cope with low pH conditions during their transit through stomach and anterior segments of the small intestine. In the present study (Chapter 5), wildtype EPEC were found to produce remarkable GABA levels *in vitro* (81.2 ± 10.3 µmol/l). Similar to eukaryotic cells, bacterial GABA is synthesized through the decarboxylation of glutamic acid or glutamine by the bacterial isoforms of the glutamic acid decarboxylase GAD A and GAD B, which differ in only five amino acids (De Biase, Tramonti et al. 1999, De Biase and Pennacchietti 2012). The respective genes, *gadA* and *gadB* are located on the EPEC chromosome. The gene *gadB* is usually transcribed in a transcriptional unit (*gadBC)* with the downstream gene *gadC* that encodes for a GABA-glutamate antiporter. Transcription of *gadA* and *gadBC* is regulated by the transcription factor GadX, which increases the expression of both enzymes in case of acidic environmental conditions (Tramonti, Visca et al. 2002). When GABA is released into the environment by the GABA-glutamate antiporter, the bacterium stabilizes its inner pH milieu by letting glutamate react with a proton before it is decarboxylated to GABA. Subsequently, GABA and a carbon dioxide molecule are released by the bacterium which factually removes a proton from the intracellular space (Tramonti, Visca et al. 2002).

Interestingly, *gadX* activation by acidic pH conditions does not only lead to an increase in GABA production via GAD A/B expression, but simultaneously also inhibits virulence gene expression, thereby lowering the attaching ability of the bacteria (Franzin and Sircili 2015). Hence, bacterial GABA metabolism does affect EPEC virulence and might also contribute significantly to the enteral GABA content.

2.4. Bacteria-Host Interaction

2.4.1. Impact of the Intestinal Microbiota on the Development of the Host's Immune System

It is well known that the gut microbiota has a profound impact on the development of a functional immune system. In adulthood, the bacterial cell load in the intestine amounts to 10^{11}-10^{12} microbes/ml luminal content, outnumbering the quantity of host cells ≥10-fold (Wall, Ross et al. 2009). During the maturation process of the infant immune system, the colonization with

various commensals starting immediately after birth is modulating the host's immune system, while the host, in turn, is essential in shaping the development of its microbial ecosystem (Hansen, Nielsen et al. 2012).

In humans and animals, the weaning process significantly determines the intestinal bacteria setup, which is vital for the future symbiosis of microbiota and host. Therefore, the intestinal establishment of a healthy microbiome plays a crucial role in the development of an efficient immune system, which has been demonstrated in several studies. For example, new-borns are initially colonized by the maternal faecal and vaginal microbiota. However, in case of caesarean section, the composition of the gut microbiota is determined by maternal skin microbiota and bacteria from the hospital environment. In the gut flora of vaginally born infants, species such as *Bifidobacteria*, *Bacteroides* and *Lactobacilli* were predominantly found, whereas infants delivered by caesarean section harboured bacterial communities of *Staphylococcus* or *Corynebacterium*. The different composition of the gut microbiota is believed to have a strong impact on immunological functions in the infant (Penders, Thijs et al. 2006, Huurre, Kalliomaki et al. 2008, Dominguez-Bello, Costello et al. 2010), influencing T lymphocyte maturation (Mazmanian, Liu et al. 2005, Mold, Michaelsson et al. 2008) or resistance to pathogen colonization (Wells 1990, Boullier, Nougayrede et al. 2003).

During weaning, the composition of the microbiome undergoes several shifts until it becomes as complex and stable as the microbiota of adults. Thus, the maturation process of the gut microbiota contributes significantly to the high susceptibility to infections during the weaning period (Fallani, Amarri et al. 2011, Koenig, Spor et al. 2011). Even though development and diversification of the microbiota continues after weaning as it is further influenced by diet and environment (Yatsunenko, Rey et al. 2012), it remains stable to a large extent throughout the adulthood, unless the interplay is disarranged by disruptive factors such as inflammation (Patterson, Cryan et al. 2014). Antibiotic treatments are also known to interfere with the sensitive relationship of the bacteria-host symbiosis (Hussey, Wall et al. 2011, Fouhy, Guinane et al. 2012), which is determined by several interactions including the interplay between host and microbes, microbes and environment, host and environment and also between different microbes. However, while it is clear that microbiota and host are influencing each other, it remains unclear how this process works in detail. The bidirectional communication system of bacteria and host is not well understood yet, but several studies suggest that microbial metabolites – such as GABA – play a crucial role as signalling molecules (Patterson, Cryan et al. 2014).

2.4.2. Impact of Microbial Metabolites

Metabolites of enteric bacteria, also called 'pharmabiotics', are often considered to act as signalling molecules communicating not only with other microbes but also with the host. Several studies indicate that pharmabiotics, including molecules such as bioactive lipids, exopolysaccharides, vitamins or amino acid derivatives like GABA or dopamine, may affect liver, brain and also intestinal functions (Patterson, Cryan et al. 2014).

Commensal bacteria have been shown to produce not only Vitamin K and B (Said 2011), but also conjugated fatty acid derivatives such as conjugated linoleic acids (CLA) (Barrett, Fitzgerald et al. 2012, Hennessy, Barrett et al. 2012) and short chain fatty acids (SCFA) (Wall, Marques et al. 2012). In human diets, CLA derives mainly from meat and milk products and are believed to have anticarcinogenic, antiobese, antidiabetic and antihypertensive properties (Koba and Yanagita 2014). *Bifidobacteria* are a main source of CLA, a metabolite resulting from the degradation of polyunsaturated fatty acids. Since it was shown that CLA levels in various tissues of pigs and mice were increased after oral administration of CLA-producing microorganisms (Wall, Ross et al. 2009), the beneficial effect of strains like *Bifidobacteria* might at least to some extent depend on their ability to produce CLA. It is believed that CLA affect different cellular pathways influencing gene expression, signal transduction and apoptosis. (Ochoa, Farquharson et al. 2004).

The microbial production of exopolysaccharides can also affect the host's immune system. Probiotics such as lactic acid bacteria are a main source of exopolysaccharides including kefiran, which has been shown to induce cytokine production and modify macrophage and splenocyte functions (Kitazawa, Itoh et al. 1996, Sato, Nishimura-Uemura et al. 2004, Vinderola, Perdigon et al. 2006).

Besides the above-mentioned bacterial degradation products, other metabolites have recently attracted attention: certain bacterial strains are able to produce neurotransmitters or their precursors in noticeable quantities. In mice, enteric bacteria were found to synthesize norepinephrine and dopamine (Tsavkelova, Botvinko et al. 2000), whereas some *Lactobacilli* are able to produce high levels of GABA (Komatsuzaki, Nakamura et al. 2008). Some authors suggest that these neurotransmitters could be involved in the neural signalling between brain and gut (Collins, Surette et al. 2012). It has also been described that the commensal microbiota can influence the serotonergic system of the host by modulating its tryptophan metabolism (Forsythe, Sudo et al. 2010). There is some evidence, that by producing neuronal active

metabolites such as serotonin, GABA and catecholamines, the microbiota might impact on the mental health of the host (Evrensel and Ceylan 2015).

2.4.3. Impact of the Microbiota on the Gut-Brain Axis

It is well known that emotions including stress or anxiety can influence intestinal functions, mainly by an intraluminal secretion of neurotransmitters leading to symptoms such as nausea or diarrhoea (Farmer, Randall et al. 2014). More recently, it turned out that the gut-brain axis is a bidirectional system, meaning that the gut and the microbiota itself modulate brain functions such as behaviour and mood (Farmer, Randall et al. 2014, Howland 2015).

In various studies, the microbiome has been shown to affect the host´s behaviour (Vuong, Yano et al. 2017). The most obvious example is the induction of sickness behaviour by pathogenic bacteria and respective LPS accumulation. The resulting depression, anxiety or reduction of feed intake is most likely mediated by cytokines such as TNFα. Vagal afferents seem to sense the accumulation of pro-inflammatory cytokines and pass this information to the brain. Subsequently, the vagal efferents trigger a release of neurotransmitters, which interferes with the inflammatory process (Goehler, Gaykema et al. 2000). In a study by Bercik et al., it has been shown that alterations in the microbiota (e.g. upon application of antibiotics) can lead to significant changes in the exploratory behaviour of mice. The same treatment did not induce any differences in behaviour patterns of germ-free mice implying a significant role of the microbiota (Bercik, Denou et al. 2011). However, the general role of probiotics on social behaviour is still discussed controversially since behavioural effects are not always present (Vuong, Yano et al. 2017). Nevertheless, several authors predict that the microbiome will become a promising target for the prevention or treatment of neuropsychiatric disorders (Dinan, Stanton et al. 2013, Cenit, Sanz et al. 2017, Kim and Shin 2017).

How the microbiome exerts its potential effects on the gut-brain axis is still a matter of debate but it is believed that the vagus nerve plays a crucial role (Forsythe, Bienenstock et al. 2014). For instance, the supplementation of a combination of *Bifidobacteria* and *Lactobacilli* has been shown to reduce anxiety-like behaviour in humans and rats (Messaoudi, Lalonde et al. 2011). In another study, however, it could be demonstrated that vagotomised mice do not display such behaviour changes upon *Bifidobacteria* supplementation suggesting a crucial involvement of the vagal system (Bercik, Park et al. 2011).
Vagal innervations appear to be most prominent in the small intestine but are also significant in the large intestine. Vagal afferents do not reach directly into the intestinal lumen, nor do they

cross the basal membrane, but instead end in the lamina propria (Wang and Powley 2007, Forsythe, Bienenstock et al. 2014). Hence, an intermediate step is required to transduce information about a chemical stimulus from the intestinal lumen to the vagal afferents. It is believed that such information is translated via sensory receptors within the epithelial cell layer. It is known that a variety of sensory receptors such as chemical receptors, mechanoreceptors, thermoreceptors and osmoreceptors are expressed by cells in the epithelial layer including enterochromaffin cells (Bellono, Bayrer et al. 2017). Chemoreceptors have been shown to be targets of gut regulatory peptides and hormones such as ghrelin, peptide YY and cholecystokinin, which influence feed/food intake and satiety. Upon chemical stimulation, enteroendocrine cells release neurotransmitters such as acetylcholine; the latter is believed to directly activate vagal afferents or other sensory fibres of visceral afferents within the epithelial cell layer. The majority of sensory fibres that have been identified in the epithelium are intrinsic primary afferents neurons (IPAN). Notably, while the bodies of typical afferent neurons are usually found in the CNS, the cell bodies of IPAN are present in the mucosa. IPAN have been shown to form self-reinforcing networks and to communicate with motoneurons as well as interneurons, thereby presenting an important part of the ENS (Furness, Kunze et al. 1998, Furness, Jones et al. 2004). It has been demonstrated, that the excitability of IPAN is affected by metabolites of *Lactobacillus rhamnosus* (*L. rhamnosus*) and *Bacteroides fragilis* (Mao, Kasper et al. 2013). Also GABA was found to have an impact on IPAN (Schlichter, Desarmenien et al. 1987). The ENS was long believed to be autonomous but nowadays there is strong evidence that there is a considerable interaction between the ENS and the vagus nerve, suggesting a potential relation of the ENS and the gut-brain axis (Powley 2000, Holzer, Michl et al. 2001). In conclusion, it seems likely that the intestinal microbiota can affect the brain by both pathways, either by influencing the vagal afferents of the gut mucosa directly or indirectly via the ENS.

In order to clarify the physiological role of the microbiota on the gut-brain axis in more detail, more information about the bacterial composition of healthy subjects is required. Although crucial improvements in diagnostic methods (e.g. next generation sequencing) have advanced the elucidation of the microbial composition significantly, microbiome research is still in its infancy. Hence, further research is necessary to identify the most important players of the gut-brain axis.

2.5. Relevance for human and animal health

The experiments of this work were conducted using EPEC isolated from diarrhoeic pigs, porcine jejunal epithelia and an intestinal porcine epithelial cell line. Since PWD is one of the most important diseases in pig breeding (Chapter 2.5.1), the results of this work are supposed to be valuable to for the understanding of PWD pathomechanisms, the epithelial response of the pig´s intestine and potential prevention strategies. However, the general findings and conclusions might be relevant for both human and animal health.

On the one hand, porcine intestinal mucosa is commonly used as a model to study intestinal physiology and pathology in man (Gonzalez, Moeser et al. 2015). It is assumed that the role of GABA in the porcine and the human gut is comparable, and findings are valid for both human and pigs. Hence, although the results of the present studies are based on experiments with porcine bacterial isolates and tissues, implications for the treatment and prevention of gastrointestinal disorders in man are likely.

On the other hand, EPEC are known to pose a serious risk to both human and animal health (Moura, Sircili et al. 2009). In piglets, EPEC can be involved in the outbreak of PWD, which is one of the most important diseases in pig farming (Chapter 2.5.1). In humans, EPEC infections are known to contribute to the occurrence of diarrhoea (Singh and Aijaz 2015) which is still one of the most common causes for mortality and morbidity worldwide (Fagundes-Neto 2013, Kotloff, Nataro et al. 2013, DuPont 2016). Particularly children in low-income countries are affected by severe cases of multifactorially induced, persistent diarrhoea, which often leads to death (Abba, Sinfield et al. 2009, Lanata, Fischer-Walker et al. 2013). Studies evaluating the etiology of diarrhoea report a prevalence of EPEC between 5-20% (Alikhani, Mirsalehian et al. 2006, Araujo, Tabarelli et al. 2007, Ochoa and Contreras 2011, Ochoa, Mercado et al. 2011). The pathomechanisms of porcine and human EPEC as well as onset and course of infection is nearly identical. Hence, the results of this work may be seen in a translational context with implications for both human and animal health.

Furthermore, the general conclusions about EPEC might be even transferable to other pathogens such as EHEC, which have been frequently detected in diarrhoeic piglets (Janke, Francis et al. 1989, Zhu, Harel et al. 1994, Osek 2002) but are also common in man (Yang, Lin et al. 2017). Since EHEC virulence is regulated very similarly as it also depends on the LEE and GadX-regulation (Branchu, Matrat et al. 2014), findings from these experiments about EPEC might also have implications for EHEC infections.

2.5.1. Postweaning diarrhoea of pigs and current treatment strategies

In piglets, EPEC can be involved in the outbreak of PWD, which is a major cause of death (Fairbrother, Nadeau et al. 2005, Vidotto, Florian et al. 2013). The disease is multifactorially induced. During the weaning period, the piglet necessarily undergoes dietary changes as well as social stress, both of which can induce intestinal inflammation. Furthermore, the diminishing maternal antibodies after weaning lead to weakened immunological protection of the mucosal barrier, which increases the intestinal susceptibility to pathogens. Antibody deficit, decreased feed intake and resulting reduction of energy supply and histological changes in the small intestine provide a gateway for pathogens (Fairbrother, Nadeau et al. 2005).

According to a recent market research, 15-23% of the piglets in European countries are affected by PWD (Elanco Animal Health 2014). Current treatment strategies are mainly based on the application of antibiotics and zinc. One of the most widely used antibiotics is colistin, which is a cationic antibiotic and is usually administered orally. It is known for its strong systemic toxicity but due to its poor absorption, it mainly accumulates in the gastrointestinal tract without entering the blood circulation of the pig. Colistin is commonly used because it is highly effective in eliminating pathogenic *Enterobacteriaceae* and has a withdrawal period of only one day after the application (Union 2010). Despite the effectiveness in the treatment of PWD, colistin is currently under scrutiny by the European Food Safety Authority (EFSA) as well as the European Medicines Agency (EMA) due to the rising bacterial resistance against this drug. In some countries, colistin resistance was detected in up to 35% of *E. coli* isolates from animals (Harada, Asai et al. 2005). Since it was discovered that colistin resistance can also be horizontally transferred (Nordmann and Poirel 2016), the use of colistin for veterinary applications is expected to be further restricted by the agencies in the near future.

The unavailability of colistin will lead to severe problems for the swine industry since other similarly effective antibiotics such as gentamycin are not suitable due to their significant withdrawal periods of up to 146 days (European Comission 2017). Furthermore, the use of zinc, which is a feasible alternative for the treatment of PWD, might be restricted as well because zinc supplementation has been shown to increase antibiotic resistance in *E.coli* (Bednorz, Oelgeschlager et al. 2013). In fact, the EFSA has recently reduced the maximum contents in feed in order to decrease the zinc emission (Authority 2014). Hence, the treatment of PWD might become even more challenging in the near future.

In the past, EPEC was frequently detected in diarrhoeic piglets (Janke, Francis et al. 1989, Zhu, Harel et al. 1994, Osek 2002). Admittedly, more recently, it was proposed that enterotoxigenic *E. coli* (ETEC) rather than EPEC are the most prevalent strain in PWD (Fairbrother, Nadeau et al. 2005, Luppi, Gibellini et al. 2016). However, the available data about the prevalence of different *E. coli* strains and their involvement in PWD outbreaks on farms should be interpreted cautiously. In most of the veterinary diagnostic laboratories, the current standard routine in PWD diagnostics only includes a screening for ETEC but not for EPEC isolates. Accordingly, the actual prevalence of EPEC in PWD might be underestimated just because it is often overlooked (Fairbrother and Gyles 2012, Rhouma, Fairbrother et al. 2017). Hence, the relevance of EPEC and their role in PWD might be even greater than currently assumed.

The high incidence of PWD, the limited therapeutic options and the fact that pathogenic *E. coli* strains are often zoonotic result in an urgent need for new prevention and treatment strategies.

3. Aim of the Study

Various effects of GABA within the CNS have been studied over the past decades. However, the role of GABA in peripheral tissues is not fully understood. Particularly the influence of GABA on the intestinal epithelium remains unclear. Enteric bacteria are a main source of GABA in the gut and the interaction between bacterial GABA production and the host´s response could play an important role in diarrhoeic diseases. In particular the transcription factor GadX which links the expression of major virulence genes with GABA production in EPEC seems to be a promising target for treatment and prevention of EPEC-induced diarrhoea.

Therefore, the aim of this work was to:

a) prove that an activation of the transcription factor GadX in EPEC would increase GABA production
b) test whether an overexpression of the transcription factor *gadX* in EPEC would lead to reduced virulence factor gene expression and reduced factual virulence *in vitro*
c) examine whether topical GABA application would have a positive effect on the mucosal barrier of the intestinal epithelium of pigs *ex vivo*, thereby potentially decreasing the susceptibility to gut infections
d) place the results in a broader, translational context with respect to their relevance for future approaches to fight diarrhoea.

4. GABA selectively increases mucin-1 expression in isolated pig jejunum.

Braun, H. S., G. Sponder, R. Pieper, J. R. Aschenbach and C. Deiner (2015). "GABA selectively increases mucin-1 expression in isolated pig jejunum." Genes Nutr 10(6): 47.

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Abstract

The inhibitory neurotransmitter GABA (γ-aminobutyric acid) is synthesized by glutamic acid decarboxylase (GAD), which is expressed in the central nervous system and in various other tissues including the intestine. Moreover, GABA can be ingested in vegetarian diets or produced by bacterial commensals in the gastrointestinal tract. As previous studies in lung have suggested a link between locally increased GABA availability and mucin 5AC production, the present study sought to test whether the presence or lack of GABA (and its precursor glutamine) has an effect on intestinal mucin expression. Porcine jejunum epithelial preparations were incubated with two different amounts of GABA or glutamine on the mucosal side for four hours, and changes in the relative gene expression of seven different mucins, enzymes involved in mucin shedding, GABA B receptor, enzymes involved in glutamine/GABA metabolism, glutathion peroxidase 2 (GPx2), and interleukin 10 (IL10) were examined by quantitative PCR (TaqMan® assays). Protein expression of mucin-1 (MUC1) was analyzed by Western blot. On the RNA level, only MUC1 was significantly up-regulated by both GABA concentrations compared with the control. Glutamine-treated groups showed the same trend. On the protein level, all treatment groups showed a significantly higher MUC1 expression than the control group. We conclude that GABA selectively increases the expression of MUC1, a cell surface mucin that prevents the adhesion of microorganisms, because of its size and negative charge, and therefore propose that the well-described positive effects of glutamine on enterocytes and intestinal integrity are partly attributable to effects of its metabolite GABA.

Keywords

GABA, glutamine, glutamic acid decarboxylase, mucin, gut health

Introduction

The non-protein amino acid γ-aminobutyric acid (GABA) acts as an inhibitory neurotransmitter exhibiting sedative, antiepileptic, anxiolytic, pain killing, hypertension lowering, and muscle relaxing properties (Rudolph et al. 1999). Accordingly, current GABA pharmacology comprises selective hypnotics, non-sedative anxiolytics, memory enhancers, and powerful analgesics acting predominantly on central GABA receptors. Providers of functional foods containing high concentrations of GABA (e. g., GABA tea, fermented brown rice, GABA-rich soy sauce, and dairy products) have jumped on this bandwagon and claim effects such as the reduction of anxiety, promotion of sleep, or action as natural tranquilizers (Cheng and Tsai 2009; Okada et al. 2000). However, reports of beneficial effects after the consumption of such foods need cautious interpretation. This is based on our current understanding that GABA acting in the central nervous system (CNS) is exclusively synthesized there (Kuriyama and Sze 1971), because the zwitterion GABA is considered to be poorly permeable across the blood-brain barrier (Goldberg 2010). Synthesis of GABA is accomplished in brain and other tissues (e.g., gastrointestinal tract) by the enzyme glutamic acid decarboxylase (GAD) by the decarboxylation of glutamate (see Fig. 1), which, in turn, can be generated by the enzyme glutaminase (GLS) through the desamination of glutamine (Pinkus and Windmueller 1977). Isoforms of GAD are widely distributed throughout the animal and plant kingdom, from cockroach (Baxter and Torralba 1975) to *Lactobacillus* (Ueno et al. 1997) and from *Escherichia coli* (Fonda 1972) to tomato (Akihiro et al. 2008) and barley (Inatomi and Slaughter 1975). Accordingly, GABA is a natural component of the free amino acid pool of all kinds of ingested plants and probiotic/commensal bacteria in the gastrointestinal tract. Furthermore, the intestinal wall of the host is also able to produce GABA (Li et al. 2012; Wang et al. 2004).

Experiments with mice have revealed that the ingestion of *Lactobacillus rhamnosus* (JB-1) can modify the expression level of GABA B and A receptors in some areas of the brain leading to increased stress resistance and anxiolysis (Bravo et al. 2011). The channel of communication between the gut and the CNS is proposed to be the vagus nerve (Bravo et al. 2011; Bravo et al. 2012). Unfortunately, the molecular signal or product of the probiotic strain responsible for the effect on the GABAergic system (i.e., was it GABA or something else?) and the underlying molecular mechanisms remained unidentified.

In the present study, we have sought to test whether a tangible positive effect of GABA on its first and foremost interaction partner, namely the gut epithelium, can be identified. The epithelium is responsible for the production of mucins which are an important element of the

innate gut immune system and consist of secreted mucin glycoproteins and attached mucins in the epithelium surface glycocalyx (McGuckin et al. 2011). Proceeding from previous results in airway epithelium of healthy smokers in whom a positive correlation had been described between mucin 5AC and GAD expression (Wang et al. 2010), we hypothesized a link between locally increased GABA availability and mucin production of the intestinal epithelium. To test this hypothesis, porcine jejunum was chosen as a model, because the small intestine is provided with the thinnest mucus layer (McGuckin et al. 2011) and should, therefore, benefit most from positive effects of GABA on mucus formation. Hence, we incubated isolated porcine jejunum epithelium with various concentrations of GABA and examined mucin expression, plus the expression of enzymes involved in mucin shedding, enzymes involved in GABA metabolism, anti-inflammatory interleukin 10 (IL10) and glutathione peroxidase 2 (GPx2). We further investigated whether the effects of GABA could be mimicked by application of its precursor glutamine. The latter amino acid is critical for several enterocyte functions and most of such glutamine effects have been linked to its metabolism by the intestine (Reeds and Burrin 2001). To select appropriate GABA concentrations for *ex vivo* experiments, we initially determined the natural GABA content in the digesta of different GI segments of slaughtered pigs. For comparison, the glutamine concentration of 1 mM was chosen based on previous measurements of the physiological glutamine concentration in the jejunal lumen of weaned piglets (Wang et al. 2008).

Materials and Methods

GABA measurements in digesta

To determine the normal GABA levels in the pig intestine, digesta samples of stomach, duodenum, mid jejunum, caecum, and mid colon of six pigs were taken immediately after slaughter at a local abattoir. The fluid phase of the digesta was extracted by centrifugation at 15,000 g for 15 minutes at 0°C, precipitated with 0.2 M perchloric acid 1: 1 and sent for HPLC analysis (Knauer GmbH, Berlin, Germany). Ultra-high-pressure liquid chromatography with electrospray ionization and mass spectrometry (UHPLC-ESI-MS) was performed by using 6-aminoquinolyl-N-hydrosysuccinimidyl carbamate (AQC) as a pre-column derivatization reagent (Cohen and Michaud 1993). Samples of the feed that pigs had received during the weeks before slaughter were homogenized in chromatography water (LiChrosolv, Merck Millipore,

Darmstadt, Germany) at a ratio of 1 g : 3.5 ml and processed accordingly (for feed composition see Supplementary Table 1).

Animals for *ex vivo* determination of GABA effects

The protocol was approved by the local authorities (LAGeSo Berlin; T 0301/11). Three fattening pigs (one male, two female) at a weight of 28 kg were purchased from a local growth-finishing farm. Pigs were sedated by intramuscular injection of 25 mg ketamine (Ursotamin®, Serumwerk Bernburg AG, Germany) and 4 mg azaperone (Stresnil®, Janssen-Cilag, Neuss, Germany) per kg bodyweight and then killed by intracardial injection of 0.5 ml/kg T61® (Intervet, Unterschleißheim, Germany). The jejunum was immediately removed from the abdominal cavity, rinsed and stripped off from its outer muscle layers. The mucosal epithelium was placed in pre-warmed buffer solution (ingredients in mM: 105.0 NaCl, 25.0 $NaHCO_3$, 0.96 KH_2PO_4, 2.0 K_2HPO_4, 10.0 glucose, 4.0 2-N-morpholinoethanesulfonic acid (MES), 1.5 $CaCl_2$, 1.0 $MgCl_2$; pH 7.4), gassed with carbogen (95% O_2, 5% CO_2), and taken to the laboratory.

Ussing chamber set-up

From each pig, 15 pieces of epithelial preparations were mounted between the halves of conventional Ussing chambers (Martens et al. 1987) to allow for the separate incubation of the mucosal and the serosal sides and to be able to monitor tissue vitality during the experiment by measuring tissue conductance (G_T value). The tissue was bathed on both sides with 16 ml buffer solution (for composition, see previous section) at 38°C and bubbled with carbogen. After a brief equilibration period (10 min after mounting), various amounts of a stock solution containing either 100 mM GABA or 250 mM L-glutamine were added on the mucosal side to obtain four treatment groups (with three epithelia per pig): 50 µM GABA, 1.0 mM GABA, 1.0 mM L-glutamine, and 10.0 mM L-glutamine. Three chambers received no additive, and the tissue therein was incubated with buffer only to serve as a control group. To prevent osmotic effects, an osmotically equivalent amount of mannitol was added on the serosal side.

After 4 h of incubation, tissues were harvested, cut into pieces, and either snap-frozen in liquid nitrogen or fixed in RNAlater® (Ambion/Life Technologies, Darmstadt, Germany) for protein or RNA isolation.

Gene expression analysis

Total RNA was isolated by using a commercial kit including a DNAse digestion step (Nucleospin RNA II, Macherey&Nagel, Dueren, Germany). RNA integrity numbers (RINs) were tested by using a lab-on-a-chip technique (RNA 6000 Nano Kit, Agilent, Waldbronn, Germany). Only samples with RINs >7.0 were used for cDNA synthesis. Reverse transcription was performed with 500 ng RNA (750 ng for GAD) and an iScript® cDNA synthesis kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions, and reactions were diluted 1:10 (1:2 for GAD examination). Changes in the relative expression of mucins MUC1, MUC2, MUC3, MUC4, MUC12, MUC13, and MUC20, enzymes involved in glutamine/GABA metabolism (glutaminase, GLS; glutamic acid decarboxylase isoform 65, GAD65; 4-aminobutyrate aminotransferase, ABAT), GABA B receptor subunit 1 (GBR1), enzymes involved in mucin shedding (a disintegrin and metalloprotease 17, ADAM17, matrix metallopeptidase 14, MMP14), glutathion peroxidase 2 (GPx2), and interleukin 10 (IL10) were examined by quantitative reverse transcription polymerase chain reaction (qPCR) by means of TaqMan® assays (Applied Biosystems/Life Technologies, Darmstadt, Germany; 0.5 µl per well) or gene-specific intron flanking primers and probes synthesized by Eurofins MWG Operon, Ebersberg, Germany (for assay IDs and sequences/concentrations, see Supplementary Tables 2 and 3). Isoform 67 of GAD (GAD67), MUC5AC and GABA A receptor subunits β_2, β_3 and γ_2 were also tested but seemed not to be expressed in jejunum epithelium.

For qPCR experiments, a 40 cycle two-step PCR protocol (20 sec at 60°C and 1 sec at 95°C) was performed on a thermocycler (ViiA7, Applied Biosystems/Life Technologies) with 4.5 µl cDNA and three replicates per reaction. TaqMan® Fast Advanced Master Mix (Applied Biosystems/Life Technologies) or iTaq® Universal Probes Supermix (Bio-Rad) were used as mastermixes in assay volumes of 10 µl. Thresholds were automatically calculated by the cycler software. For data analysis, the software qbasePLUS (Biogazelle NV, Zwijnaarde, Belgium) was used to perform inter-run calibration, to determine dilution series-based gene specific amplification efficiencies, and to test for expression stability of reference genes. Of the six reference genes tested, beta-2 microglobulin (B2M) and beta actin (ACTB) were recommended for the normalization of TaqMan runs, whereas glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was suggested for runs with self-designed primers and probes. After the normalization of Cq values with the respective reference gene(s), results were scaled to the control group and exported as calibrated normalized relative quantity (CNRQ) values. Relative expression values were used for statistical analysis.

Western blots

Protein was isolated from 30 mg frozen epithelium samples homogenized in 1 ml extraction buffer containing 10 mM Tris, 140 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 1.0 mM phenylmethanesulfonyl fluoride, 1.0 mM 1,4-dithio-DL-threitol, and 3 tablets/200 ml cOmplete EDTA-free Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany). Total protein content was measured on a multimode plate reader (EnSpire®, PerkinElmer, Rodgau, Germany) by using the Pierce® 660nm Protein Assay (Thermo Scientific, Waltham, United States). Aliquots containing 200 µg of protein were loaded per lane on a 10% Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-Rad) containing a trihalo compound to induce a covalent reaction with tryptophan residues of the proteins in the gel when exposed to ultraviolet light. This enabled the blotting efficiency to be checked and allowed normalization in quantification analysis (Gilda and Gomes 2015; Gurtler et al. 2013). After semi-dry blotting, the polyvinylidene difluoride membrane (TransBlot Turbo Mini-size LF PVDF Membrane, Bio-Rad) was blocked with 5% w/v bovine serum albumin (BSA) in Tris-buffered saline + Tween (50 mM Tris, 150 mM NaCl, 0.01% Tween-20, pH 7.6) for 2 hours, and then a rabbit polyclonal anti-mucin-1 antibody (ARP41446_T100, Aviva Systems Biology, San Diego, USA) was applied at a concentration of 2.5 µg/ml for 12 hours. A 1:1000 horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (7074S, Cell Signaling Technology Inc., Danvers, USA) was used as a secondary antibody. The UV-light-activated membrane was imaged by using the ChemiDoc MP Imaging system (Bio-Rad), and the relative amount of total protein in each lane on the blot was calculated by the software Image Lab 4.1 (Bio-Rad). The total protein signals were then used for the normalization of the specific signal, which was visualized with SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, Waltham, USA). Each specific band was normalized to the blackness of the respective lane by Image Lab 4.1 (Gilda and Gomes 2015), and results were given as normalized intensities (NI). Controls were always assumed as NI = 1 by the software. However, as only one sample could be defined as being the control, NI values of treated epithelia were related to each of the three control epithelia on the same blot and then averaged, finally resulting in nine relative values per treatment group.

Statistical methods

A one-way analysis of variance (ANOVA) test was performed by using the software Sigma Plot 11.0 (Systat Software GmbH, Erkrath, Germany). Data were tested for normality (Kolmogorov-Smirnov's test with Lilliefors' correction) and equal variance (Levene's median test), and if either test failed, the Kruskal-Wallis one-way ANOVA on ranks was performed. Multiple comparisons (Dunnett's method) compared either the GABA- or the glutamine-treated groups with the control. *P*-values of < 0.05 were considered to be statistically significant.

Results

GABA measurements in digesta and pig feed

In the digesta fluid of slaughtered pigs, the highest GABA concentrations were found in the stomach (median 27.6, min. 4.8, max. 100.4 µmol/l) and then decreased in the small intestine (duodenum median 4.9, min. 3.4, max. 76.6; jejunum median 3.9, min. 1.8, max. 26.2 µmol/l; see Fig. 2). Towards the large bowel, GABA concentrations increased again (caecum median 16.5, min 12.2, max 22.8; colon median 21.9. min 9.8, max 53.2 µmol/l). One-way ANOVA showed a difference between the segments ($P = 0.047$); this difference was attributable to significantly less GABA in the jejunum compared to the stomach and the colon, with intermediate values in the duodenum and caecum. GABA levels in three samples of the aqueous extract from pig feed were 179.8 ± 11.4 µmol/l (mean ± SD).

Influence of GABA and glutamine application *ex vivo* on porcine jejunal epithelium

Epithelia of all groups showed stable G_T values for the first 3 h of incubation with only slight increases towards the 4th hour, indicating good tissue vitality (data not shown).

Gene expression analysis

Relative expression data of MUC1 expression showed significant differences between both GABA concentrations and the control group (Fig. 3 and Table 1). Both glutamine-treated groups also showed a trend for MUC1 mRNA up-regulation ($P = 0.14$). Other genes and mucins tested were not affected by the treatment (Table 1).

Western blot

As MUC1 gene expression was significantly affected by the treatment, MUC1 expression was verified on the protein level by Western blot (Fig. 4) and subsequent quantification (Fig. 5). On the protein level, all treatment groups showed a significantly higher MUC1 expression than the control group ($P < 0.05$).

Discussion

The present study was initiated to test the hypothesis as to whether GABA is a novel signaling molecule for immune functions in the gastrointestinal tract. This hypothesis was derived from a finding in airway epithelium in which continuous irritating stimuli (smoking) induced an up-regulation of the GABA-producing enzyme GAD67 coinciding with an increased production of MUC5AC (Wang et al. 2010). In the present study, we were able to show that dosages of luminal GABA in the upper range or exceeding the concentrations in porcine intestine induced a selective up-regulation of MUC1 at both the mRNA and protein levels. Thereby, we demonstrated, for the first time, a causal relationship between GABA and mucin production. With special reference to the intestine, this implies that GABA ingested with food or feed and GABA produced by the epithelium or indigenous bacteria in the gastrointestinal tract can have a direct and very specific stimulatory effect on a key component of the innate immune system. An analogy between the effects of GABA and glutamine further suggests that the endogenous synthesis of GABA upon glutamine exposure can likewise increase the resistance of the gut to infectious agents by up-regulating MUC1 expression.

The observation of McGuckin et al. that the mucus layer is at its thickest in the stomach and colon (McGuckin et al. 2011) apparently not only correlates with the number and distribution of mucus producing cells throughout the gastrointestinal tract but also with our finding that GABA levels are highest in the stomach and colon. The GABA in the stomach seems to be derived from the pig feed, which evidently contains high levels of GABA. The drop of luminal GABA concentration in the small intestine was anticipated based on the efficient GABA absorption observed in previous studies in Caco-2 cells (Thwaites et al. 2000). The subsequent rise of luminal GABA towards the hindgut might be a result of water absorption in the large intestine, leading to a concentration of non-absorbed luminal GABA, but might also imply that new GABA is produced and released in the hindgut. The latter might occur as a result of the action of either the glutamic acid decarboxylases of gut microbiota or the endogenous GAD of

the intestinal epithelium. Of these two options, bacteria as the major source are more likely, because endogenous GABA production is restricted to selected endocrine cells within the epithelium and to neurons that have been proposed to play a role in tissue maturation and differentiation (Gilon et al. 1987; Wang et al. 2006). Moreover, both commensal and potentially pathogenic bacteria are well known for their ability to synthesize and release large amounts of GABA especially under acidic stress to remove cytoplasmic protons (Fonda 1972; Gorden and Small 1993; Hersh et al. 1996; Ueno et al. 1997). Under the assumption that bacterial metabolites influence the expression of mucus and its components, respectively, the observation that germfree rodents are provided with less cecal goblet cells and non-goblet mucous-type cells than conventionally raised/conventionalized rodents (Kandori et al. 1996; Ishikawa et al. 1989) becomes plausible.

The present study suggests that GABA incorporated with the food or produced inside the gut has significant local effects in the intestine, namely, a selective up-regulation of MUC1. The MUC1 protein is the best characterized transmembrane mucin and is an important player of intestinal defense. For example, deficiency in MUC1 increases the susceptibility to infections by *Campylobacter jejuni* (McAuley et al. 2007) and *Helicobacter pylori* (McGuckin et al. 2007). MUC1 is translated as a single polypeptide that is then cleaved in the endoplasmic reticulum. The C-terminal domain anchors the protein to the cell surface and acts in signal transduction. In growth-factor-triggered signaling pathways, this domain interacts with transcription factors, thereby driving the expression of genes involved in changes of the cytoskeleton and the adhesive capacity of the cell.

The extracellular domain of MUC1 has an extended structure with a large number of sialylated *O*-glycans. Chemically, sialic acids are nine-carbon monosaccharides and represent highly electronegative residues that influence the tertiary structure of proteins and protect them against proteolysis. In addition, they also act as binding partners for lectins. A surface lectin of macrophages has been shown to be able to bind MUC1 and to initiate signal transduction leading to cell growth and altered cell-cell adhesion (Tanida et al. 2013). Evidence has also been presented that a variety of lectins (not only lectins of immune cells) bind to mucins, e.g., wheat germ agglutinin (Jeffers et al. 2010) and parasite adherence lectin (Chadee et al. 1987). We can, therefore, assume that mucins generally bind lectins, including toxic/harmful lectins from plants/legumes, fungi, bacteria, and viruses. Although doubts remain as to whether the binding of lectins always leads to signal transduction by MUC1, the detaining of alien

molecules by binding them to carbohydrate moieties of the mucin layer is an important part of mucosal defense strategy.

The degree of sialylation and, hence, the resulting lectin binding capacity are regulated by enzymes including sialidases (i.e., neuraminidases) which are also found in bacteria (Gaskell et al. 1995). The pH optimum of bacterial sialidases is ~5-7 (Corfield et al. 1981), which would permit mucin desialylation by bacteria in the gut from the duodenum to colon. Desialylation by bacteria would facilitate the digestion of the mucin protein core and subsequent invasion of the epithelium. In such a scenario, GABA concentrations could serve the epithelium as an indirect measure for luminal bacterial loads. On the assumption that increasing bacterial loads increase the risk of MUC1 protein degradation and epithelial infection, an enhanced MUC1 expression by epithelial cells receiving the GABA signal could be valuable for initiating the replenishment of the protective mucus layer to compensate for mucolytic bacterial activity.

Having observed that GABA is a key signal for increasing MUC1 expression in isolated intestinal preparations, we further wished to determine whether the GABA precursor glutamine had a similar effect. Glutamine is a central amino acid in energy and nitrogen metabolism and constitutes the most abundant amino acid in the blood. It is an important fuel for enterocytes and stimulates protein synthesis (Higashiguchi et al. 1993). It is protective during intestinal inflammation (e.g., by the synthesis of heat shock protein 70 (Xue et al. 2011)) and supports healing in intestinal barrier dysfunction after severe trauma (Li et al. 2002). During the weaning phase of piglets, dietary supplementation with 1% glutamine is able to prevent jejunal atrophy; this prevention is linked to enhanced growth performance (Wu et al. 1996). However, in cell culture, glutamine is also known to be of special importance as a nutritive factor. Deficiency in glutamine can even lead to apoptosis in cells that are under the influence of the oncogene MYC. Although the reasons are not clear, apoptosis is prevented when other substrates of the Krebs cycle (e.g., pyruvate, oxaloacetate) are available (Yuneva et al. 2007). This is in accordance with the general opinion that most glutamine effects are based on the delivery of substrates for ATP production in the Krebs cycle; e.g., Yuneva et al. suggest that glutamine provides the Krebs cycle with a carbon chain, and that this chain is the backbone of other intermediates. The present study suggests that metabolic conversion to GABA constitutes a second possibility by which glutamine can elicit beneficial effects on intestinal integrity.

Compliance with Ethics Guidelines

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Hannah-Sophie Braun, Gerhard Sponder, Robert Pieper, Jörg R. Aschenbach, and Carolin Deiner declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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Figures and Tables

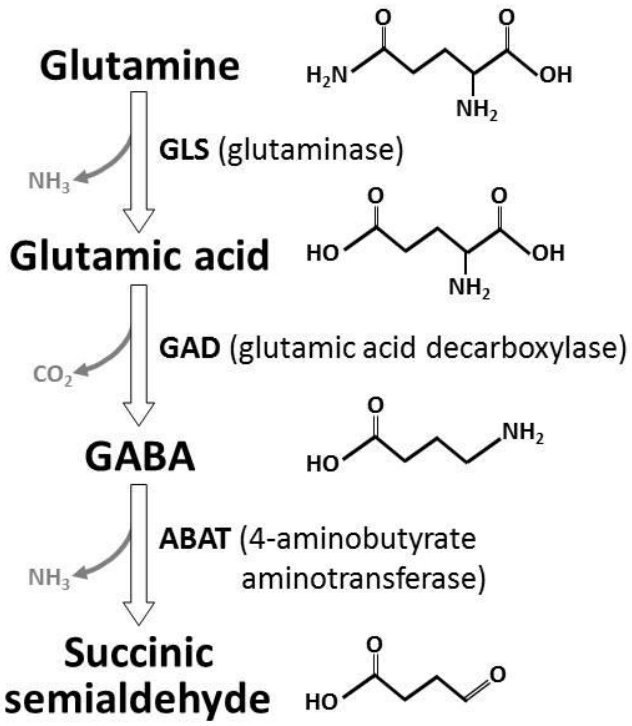

Figure 1: Enzymes involved in the metabolism of GABA.

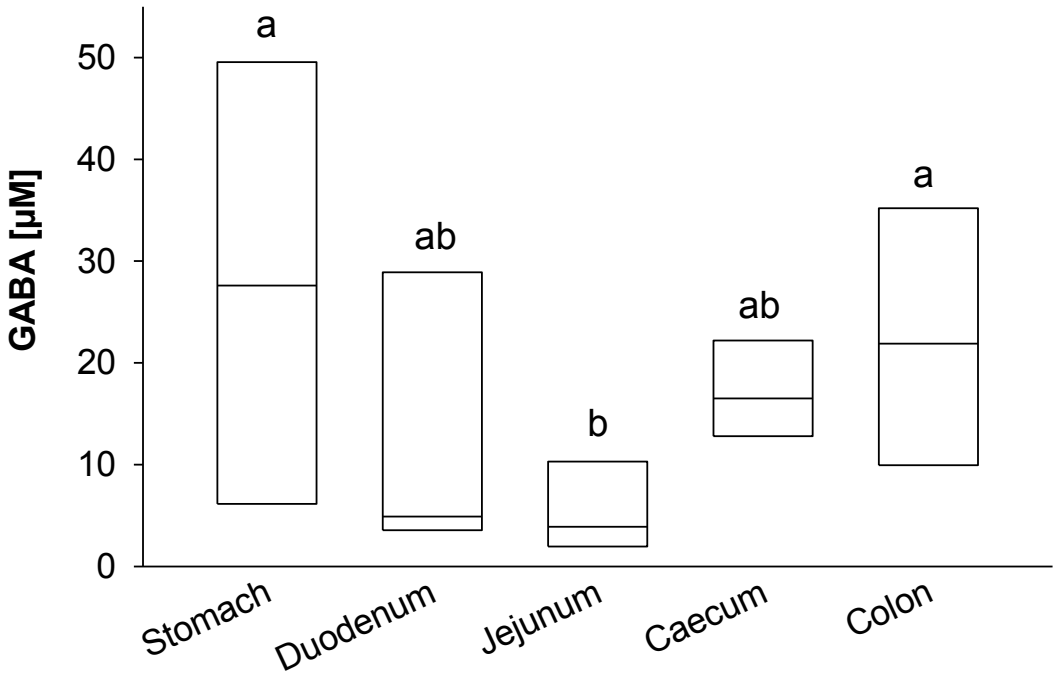

Figure 2: GABA concentrations (medians and percentiles 25/75) in the digesta of various segments of the gastrointestinal tract. Digesta samples were taken immediately after slaughter, and GABA levels were determined by UHPLC-ESI-MS. [a,b]Columns that do not share a common letter are significantly different (P = 0.036); n = 6. The GABA content in pig feed extract (produced from 1g feed in 3.5 ml ultrapure water) was 179.8 µM.

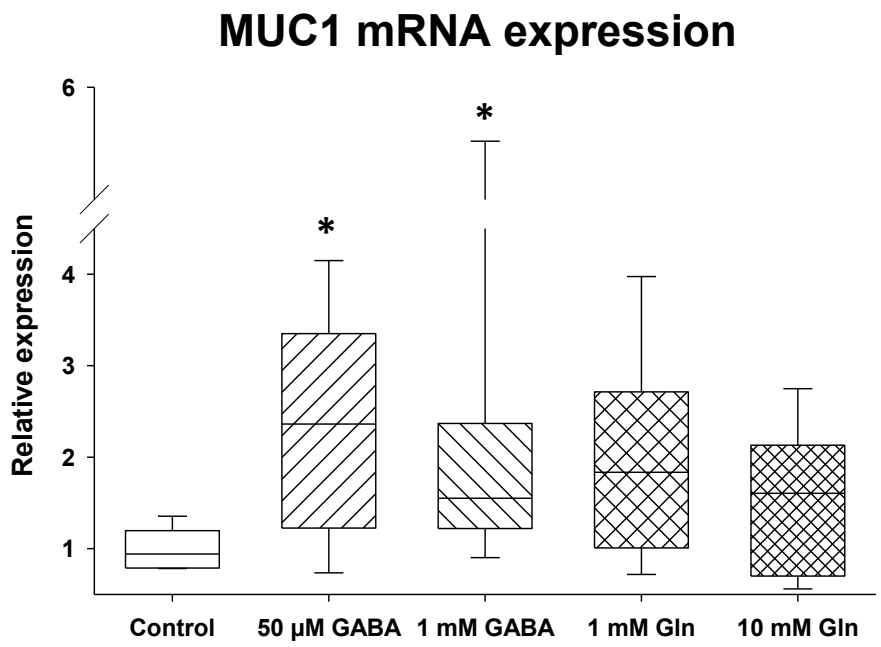

Figure 3: Relative expression levels of mucin-1 mRNA in buffer control and in GABA- or glutamine-treated jejunum tissue samples. Original data were normalized to beta-2 microglobulin and beta-actin and were calibrated to the mean of the control group. Box plots show 25/75 percentiles and medians. Asterisks indicate statistically significant differences between treatment and control ($P < 0.05$). n = 9

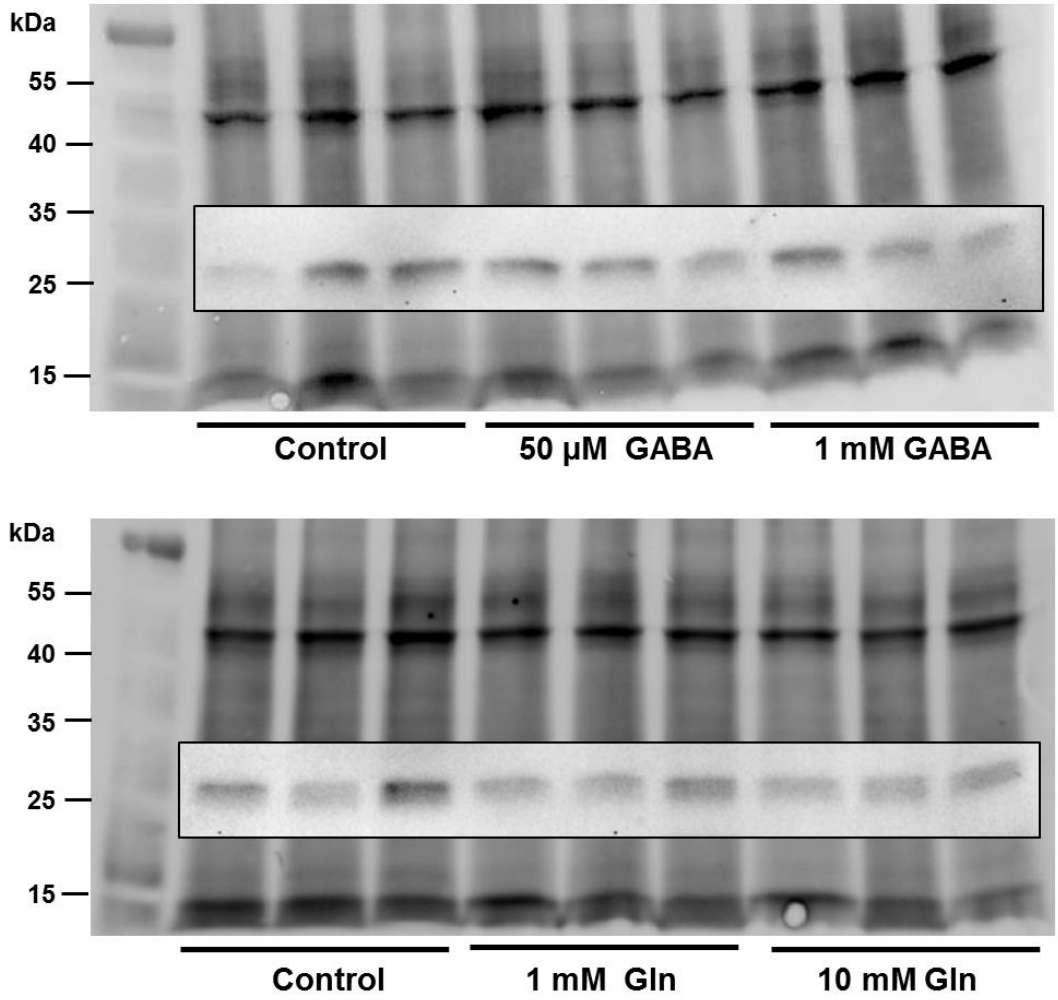

Figure 4: Representative Western blots from one pig with three samples per group. Mucin-1 protein expression is presented in the small framed picture overlying the image taken for normalization (illustrating total protein load on the gel by UV activated tryptophan residues): the intensity of each mucin-1 specific band was normalized (NI) to the blackness of its underlying total protein lane. Resulting NI values were compared to the control which was set to NI=1. MUC1 was up-regulated in all treatment groups receiving either GABA or glutamine (Gln) compared with the control tissues.

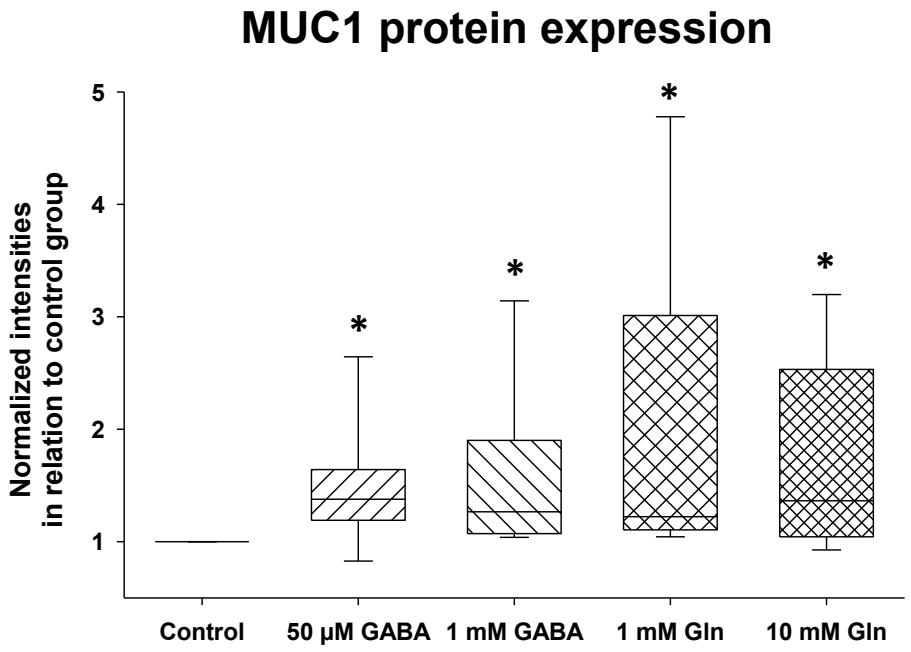

Figure 5: Relative mucin-1 protein expression in the buffer control and in GABA- or glutamine (Gln)-treated jejunum samples. Original data were normalized to total protein and scaled to controls. Box plots show 25/75 percentiles and medians. Asterisks indicate statistically significant differences between treatment and control ($P < 0.05$). n = 9

Table 1: Influence of mucosal incubation with GABA or glutamine on mRNA expression in porcine jejunum[a]

	MUC1	MUC2	MUC3	MUC4	MUC12	MUC13	MUC20
Control	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.16	1.00 ± 0.24	1.00 ± 0.23	1.00 ± 0.24	1.00 ± 0.16
50 µM GABA	**2.33 ± 0.40**	1.11 ± 0.15	0.96 ± 0.10	1.20 ± 0.21	0.56 ± 0.10	0.84 ± 0.21	0.88 ± 0.17
1 mM GABA	**2.04 ± 0.50**	1.29 ± 0.09	0.92 ± 0.11	0.90 ± 0.20	0.86 ± 0.11	0.95 ± 0.20	0.83 ± 0.12
P-value	**0.02**	0.14	0.91	0.55	0.12	0.82	0.70
Control	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.16	1.00 ± 0.24	1.00 ± 0.23	1.00 ± 0.24	1.00 ± 0.16
1 mM Gln	1.99 ± 0.36	1.07 ± 0.06	0.98 ± 0.12	1.00 ± 0.21	1.03 ± 0.26	0.96 ± 0.18	0.78 ± 0.08
10 mM Gln	1.47 ± 0.26	0.93 ± 0.10	1.27 ± 0.25	0.71 ± 0.15	0.94 ± 0.24	0.71 ± 0.15	0.82 ± 0.16
P-value	0.14	0.55	0.68	0.35	0.96	0.52	0.49

	GLS	GAD65	ABAT	GBR1	ADAM17	MMP14	GPx2	IL10
Control	1.00 ± 0.24	1.00 ± 0.09	1.00 ± 0.29	1.00 ± 0.11	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.19	1.00 ± 0.11
50 µM GABA	0.85 ± 0.21	1.48 ± 0.30	0.69 ± 0.15	1.00 ± 0.07	0.86 ± 0.24	1.00 ± 0.19	1.00 ± 0.14	1.17 ± 0.12
1 mM GABA	0.71 ± 0.11	1.14 ± 0.21	0.59 ± 0.11	1.02 ± 0.08	0.78 ± 0.15	0.84 ± 0.21	0.87 ± 0.16	1.08 ± 0.15
P-value	0.65	0.46	0.60	0.62	0.71	0.65	0.77	0.47
Control	1.00 ± 0.24	1.00 ± 0.09	1.00 ± 0.29	1.00 ± 0.11	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.19	1.00 ± 0.11
1 mM Gln	0.70 ± 0.08	1.57 ± 0.36	0.59 ± 0.08	1.01 ± 0.08	0.68 ± 0.11	0.88 ± 0.14	1.03 ± 0.20	1.13 ± 0.16
10 mM Gln	1.01 ± 0.34	1.21 ± 0.29	0.85 ± 0.22	0.83 ± 0.07	0.92 ± 0.15	1.15 ± 0.18	0.93 ± 0.18	1.13 ± 0.12
P-value	0.77	0.80	0.49	0.25	0.30	0.54	0.95	0.68

[a]Data were normalized to beta-2 microglobulin and beta-actin (MUC1, MUC2, GAD65) or GAPDH (other genes) and scaled to the mean of the control group. Values represent means ± SEM. For abbreviations, see text section "Gene expression analysis"; n = 9.

5. The GadX regulon affects virulence gene expression and adhesion of porcine enteropathogenic *Escherichia coli* in vitro.

Braun, H. S., G. Sponder, R. Pieper, J. R. Aschenbach, K. Kerner, R. Bauerfeind and C. Deiner (2017). " The GadX regulon affects virulence gene expression and adhesion of porcine enteropathogenic Escherichia coli in vitro." Veterinary and Animal Science 3: 10-17.

DOI: 10.1016/j.vas.2017.04.001

Abstract

The ability of enteropathogenic *Escherichia coli* (EPEC) to express virulence factor genes and develop attaching and effacing (AE) lesions is inhibited in acidic environmental conditions. This inhibition is due to the activation of transcription factor GadX, which upregulates expression of glutamic acid decarboxylase (Gad). Gad, in turn, produces γ-aminobutyric acid (GABA), which was recently shown to have a beneficial effect on the jejunal epithelium *in vitro* due to increased mucin-1 levels.

In the present study, we sought to test whether forced GadX activation/overexpression abolishes virulence associated features of EPEC and provokes increased GABA production. EPEC strains were isolated from diarrheic pigs and submitted to activation of GadX by acidification as well as *gadX* overexpression via an inducible expression vector plasmid. GABA concentrations in the growth medium, ability for adhesion to porcine intestinal epithelial cells (IPEC-J2) and virulence gene expression were determined.

Growth in acidified media led to increased GABA levels, upregulated *gadA/B* expression and downregulated mRNA synthesis of the bacterial adhesin *intimin*. EPEC strains transformed with the *gadX* gene produced 2.1 to 3.4-fold higher GABA levels than empty-vector controls and completely lost their ability to adhere to IPEC-J2 cells and to induce actin accumulation.

We conclude that intensified *gadX* activation can abolish the ability of EPEC to adhere to the intestinal epithelium by reducing the expression of major virulence genes.

Key words: EPEC, GadX, GABA, adhesion, bacteria-host interaction, diarrhea

1. Introduction

Piglets around weaning are known to be exceedingly susceptible for gut infections, since they undergo environmental and psychological stress as well as nutritional changes. The postweaning diarrhea, starting around 3-10 days after the piglets are weaned, has been a focus of research due to the high economic losses it entails in pig production (Fairbrother, Nadeau, & Gyles, 2005; Hampson, Woodward, & Connaughton, 1993; Tsiloyiannis, Kyriakis, Vlemmas, & Sarris, 2001).

These losses are not only caused by severe diarrhea and the resulting higher mortality but also by decreased growth performance and reduced weight gain. A characteristic pathomechanism of enteropathogenic *Escherichia coli* (EPEC) to cause diarrhea in humans and animals is the ability to provoke attaching and effacing lesions (AE lesions) (Bruant et al., 2009; DebRoy & Maddox, 2001; Girard, Batisson, Frankel, Harel, & Fairbrother, 2005; Kim, Kim, Hur, & Lee, 2010; Nataro & Kaper, 1998). AE lesions are histopathological alterations in epithelial cells of the intestine. They are characterized by the effacement of microvilli through rearrangement of the epithelial cell cytoskeleton (Kaper, McDaniel, Jarvis, & Gomez-Duarte, 1997), leading to a pedestal-forming actin accumulation directly beneath the adherent bacteria (Kalman et al., 1999; Kaper et al., 1997; Kaper, Nataro, & Mobley, 2004). The concurrent destruction of the enteric brush border results in enteric malfunction and diarrhea.

Since infections with EPEC usually occur orally, EPEC, as well as many other bacteria, have evolved a system to cope with low pH conditions using the enzyme glutamic acid decarboxylase (Gad), which converts glutamic acid into γ-aminobutyric acid (GABA). The subsequent release of GABA via a GABA-glutamate antiporter represents the factual extrusion of protons and stabilizes the bacteria's inner pH milieu (Foster, 2004; Hersh, Farooq, Barstad, Blankenhorn, & Slonczewski, 1996; Richard & Foster, 2003). In a previous study, we have shown that luminal GABA has significant local effects in the small intestine, namely a selective upregulation of mucin 1 (MUC1) (Braun, Sponder, Pieper, Aschenbach, & Deiner, 2015). The MUC1 protein is the best characterized transmembrane mucin and is an important player of intestinal defense. This implies that GABA ingested with vegetable food or produced by bacteria can have a direct stimulating effect on a key component of the mucosal barrier function.

In *E. coli* two functionally undistinguishable isoforms of Gad are known: GadA and GadB (De Biase, Tramonti, John, & Bossa, 1996; Smith, Kassam, Singh, & Elliott, 1992). Gene expression of *gadA* and *gadB* is regulated by the transcription factor *gadX*, located downstream

of *gadA*. In case of acidic environmental conditions, *gadX* expression is increased, thereby leading to an upregulation of GadA/B production (Tramonti, Visca, De Canio, Falconi, & De Biase, 2002). As the pH optimum of bacterial Gad activity is discussed to be between 3.8 and 5 (De Biase, Tramonti, Bossa, & Visca, 1999; Shukuya & Schwert, 1960), it can be speculated that the more acidic the environment (e.g. stomach, cecum), the more GABA is produced, thereby solidifying the mucosal barrier. However, growth circumstances have to be considered because the presence of the *gadA/B* gene transcripts did not necessarily correlate with Gad enzyme activity, especially when cells were grown under acidic conditions (Castanie-Cornet, Penfound, Smith, Elliott, & Foster, 1999).

In addition to its upregulating effect on the Gad system, GadX has also been shown to downregulate the expression of genes of the locus of enterocyte effacement (LEE) (Shin et al., 2001). It is widely accepted that LEE is largely responsible for the pathogenicity of EPEC as it includes almost all virulence genes necessary for the formation of AE lesions (An et al., 2000; Elliott et al., 1998; McDaniel, Jarvis, Donnenberg, & Kaper, 1995; McDaniel & Kaper, 1997; Shaw, Cleary, Murphy, Frankel, & Knutton, 2005). LEE comprises EscU, a component of the type III secretion system (T3SS), a needle-forming multiprotein complex, which spans through the inner and outer bacterial membrane and facilitates the translocation of bacterial effector molecules directly from the bacterial into the host cell cytoplasm (Jarvis et al., 1995).

Further gene loci on LEE encode for the translocated intimin receptor (Tir) and a Tir-specific chaperone (CesT). Upon its translocation into the host cell cytoplasm via the T3SS, Tir is inserted into the host cell membrane and serves as a receptor for the bacterial adhesin intimin (Kenny, 2002; Kenny et al., 1997). Intimin, an outer membrane protein, is known as a major virulence factor of EPEC as it is required for the intimate attachment of the bacterium to the host cell. Intimin is also encoded by a gene, *eae,* localized within the LEE. The *eae* gene is commonly used in diagnostic PCR procedures for the identification of EPEC (An et al., 2000; Gomez-Duarte & Kaper, 1995; Jerse, Yu, Tall, & Kaper, 1990).

The LEE-encoded regulator (Ler) activates the expression of all virulence genes of LEE. Ler itself is positively regulated by the plasmid encoded regulator (PER) (Mellies, Elliott, Sperandio, Donnenberg, & Kaper, 1999). The *per* gene, in turn, is a region located on a 90 kb plasmid, which is inhibited by GadX (Shin et al., 2001). Hence, it can be speculated that the more acidic the environment, the more GadX is activated and the more PER is inhibited. This

prevents EPEC from unnecessary expression of virulence factors in adverse environments, e.g. during their passage through the stomach.

Acidic conditions were observed to influence Gad activity (Castanie-Cornet et al., 1999). However, in the laboratory setting, acidification may irritate the hosting cell layer. Therefore, we have used acidification only as an initial proof of concept and then switched to a *gadX* overexpression model at neutral pH values. Accordingly, we used different EPEC strains isolated from diarrheic pigs, introduced an inducible expression vector plasmid containing the *gadX* gene and subsequently examined GABA production, adhesion ability *in vitro* and virulence gene expression on the mRNA level.

2. Methods

2.1 Experimental design

2.1.1 Proof of concept: influence of acidification on GABA production, *gadA/B* and *eae* expression.

One EPEC strain was grown at either pH 5.0 or pH 7.0. RNA was isolated, reversely transcribed and analyzed for the expression levels of *gad A/B* and *eae*. GABA concentrations in the medium were measured. The experiment was repeated on four consecutive days to a total of four replicates per sample.

2.1.2 Influence of *gadX* overexpression on GABA production.

One EPEC strain was transformed with either an inducible *gadX* plasmid or an empty plasmid. Two, three and four hours after induction with isopropyl-β-D-thiogalactopyranosid (IPTG), GABA concentration was measured in the culture medium (pH 7.0). According to the results, two additional EPEC strains were transformed and GABA levels measured only after two hours of induction with IPTG. The experiment was repeated on three consecutive days to a total of three replicates per sample.

2.1.3 Influence of *gadX* overexpression on virulence gene expression.

RNA of transformed porcine EPEC strains was isolated 2 hours after induction of the plasmid and analyzed for gene expression changes of *gadX* and *gadA/B* as well as of the major virulence

factors intimin, *tir*, *cesT* and *escU*. Duplicates of the experiment were repeated on four consecutive days to a total of eight replicates per sample.

2.1.4 Influence of *gadX* overexpression on adhesion ability

The ability to adhere and cause AE lesions was tested *in vitro* on intestinal porcine epithelial cells (IPEC-J2) using the fluorescent actin staining (FAS) test and a regular adhesion test with the three transformed porcine EPEC strains and their respective wild type strains (one replicate per strain).

2.1.5 pH measurements in the gastrointestinal tract.

In order to evaluate the physiological pH levels in different segments of the gastrointestinal tract, digesta samples of six pigs were analyzed using a pH meter.

2.2 Method details

2.2.1 Bacterial strains, culture conditions and transformation.

All EPEC used in the present study (Table 1) had been isolated from either diarrheic piglets or porkers, and had been checked for the presence of the intimin gene *eae*, their ability to cause actin accumulation in intestinal porcine epithelial (IPEC-J2) cells (FAS test) and their growth performance in M9 minimal medium. According to the presence or absence of the *bfpA* plasmid (Nataro & Kaper, 1998), strains belonged to the group of typical or atypical EPEC, the latter being more common in pigs (Bruant et al., 2009; Frohlicher, Krause, Zweifel, Beutin, & Stephan, 2008). Strains P185/04-2, and 4181/05-2 were characterized as atypical EPEC lacking the *bfpA* plasmid but harboring several other virulence-associated genes (e.g. non LEE virulence genes as *astA*, *irp2*, *fyuA*). Strain P6414/05-1 was the only typical EPEC available.

The standard culture medium to grow or transform bacteria was LB medium supplemented with ampicillin (0.1 g/l) at pH 7.3 and 37°C. Bacteria were transferred to M9 minimal salt medium for experiments because standard LB medium was found to contain GABA (M9 minimal salt medium: Na_2HPO_4 15.1 mmol/l, KH_2PO_5 6.6 mmol/l, NH_4Cl 18.6 mmol/l, NaCl 8.6 mmol/l, MES glucose 22.0 mmol/l, glutamate 2.0 mmol/l, $MgSO_4$ 2 mmol/l, $CaCl_2$ 0.1 mmol/l, ampicillin 0.1 g/l; pH 7.0; 37°C). For the proof of concept, the pH of M9 was adjusted to 5.0 or 7.0.

The expression vector plasmid pQE-80L (4,751 bp; Ampr; Qiagen, Hilden, Germany) was used as it includes a cis-*lacIq* gene that overexpresses the Lac repressor, strongly suppressing protein

expression from the Lac promoter unless induced with IPTG. The *gadX* encoding DNA (EcoGene Accession Number EG12243) was synthetically manufactured (Life Technologies, Darmstadt, Germany) with restriction sites for BamH1 and HindIII. T4 Ligase (Fermentas, St. Leon-Rot, Germany) was used to ligate the insert into the linearized plasmid resulting in the recombinant plasmid pQE-80L::*gadX*. Porcine EPEC were treated with 75 mM $CaCl_2$ to produce chemically competent cells, transformation was accomplished by heat shock (Sambrook & Russell, 2006). The untreated plasmid pQE-80L was used to generate strain-specific empty plasmid transformants, accordingly.

2.2.2 GABA measurements.

For GABA measurements, the OD_{600} of the respective overnight culture was determined and the volume necessary for a final culture volume of 10 ml with an OD_{600} of 0.1 was added to prewarmed M9 medium. IPTG was added. For each time point, 1 ml of the culture was taken, OD_{600} was determined for later normalization, samples were centrifuged for 15 min (at 14,000 rpm, 0°C) and 200 µl of the supernatant were precipitated with 200 µl of 0.2 M perchloric acid. Analysis of GABA was performed by Knauer GmbH (Berlin, Germany) using ultra-high performance liquid chromatography with electrospray ionization-mass spectrometry detection (Cohen & Michaud, 1993).

2.2.3 Bacterial RNA isolation and cDNA synthesis.

A volume equivalent to 1 ml with an OD_{600} of 0.2 was collected from the respective bacterial overnight culture two hours after induction with IPTG or acidification, respectively. After centrifugation for 15 min (14,000 rpm, 0°C) the supernatant was discarded and the pellet was resuspended in lysozyme according to the manufacturer's instructions for Gram-negative bacteria of the peqGOLD Bacterial RNA Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). Notwithstanding the peqGOLD protocol, lysis of cell membranes was facilitated by incubating the pellet in liquid nitrogen for 15 min before resuspension; thereafter, it was proceeded with the peqGOLD protocol including DNAse treatment.

RNA integrities and concentrations were examined by use of a lab-on-a-chip system (Prokaryote Total RNA Nano, Agilent Technologies, Waldbronn, Germany) showing RINs around 9.6. Only RNAs with RINs > 7 were used for cDNA synthesis, assuming that lower quality could affect results. RNA yields ranged from 6 to 100 ng/µl.

One hundred ng of RNA were transcribed to the reverse strand using the iScript cDNA synthesis kit (Bio-Rad Laboratories GmbH, Munich, Germany) in accordance with manufacturer`s instructions. The reaction product was diluted 1:10 and stored at -20°C until further processing.

2.2.4 Reverse transcription-quantitative PCR (RT-qPCR).

Primers were designed to detect the target genes *gadX*, *gadA* and *gadB* (genes encoding glutamic acid decarboxylases A and B, respectively), *eae*, *tir*, *cesT* and *escU* using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3, for primer sequences see Table 2). The primer set for detection of *gadA* and *gadB* was designed to detect a highly conserved region in both isoforms, hence, it is referred to as *gadA/B*. Specificity of established RT-qPCR assays was assured by electrophoresis of PCR products through 1.5% agarose gels and sequencing of amplicons (GATC Biotech, Konstanz, Germany). For normalization, reference genes *cysG* and *gapdh* (primer sequence published by (Carey, Kostrzynska, & Thompson, 2009)) were used. According to geNorm (Biogazelle NV, Zwijnaarde, Belgium), neither reference gene *rrsA* showed a sufficiently stable expression level, nor previously tested genes *hcaT* and *idnT* (Zhou et al., 2011).

Relative quantification of specific mRNA was conducted on a ViiA7 Cycler (Life Technologies, Darmstadt, Germany) according to the following protocol: 95°C for 10 min, subsequent 40 cycles at 95°C for 15 sec and 1 min at 60°C, followed by a melting curve analysis at 95°C for 15 sec, cooling down to 60°C for 1 min and heating up slowly to 95°C for 15 sec while monitoring fluorescence.

Every sample reaction was carried out in triplicates on a 384-well plate. Each 10 µl reaction contained 5 µl of cDNA, 4.64 µl of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) and 0.18 µl of forward and reverse primers (20 µmol/l). No template controls (NTC) and negative RT samples (reverse transcription omitted) were used for every target gene. Samples were excluded from evaluation if Cq difference between sample and respective NTC was < 5 or if the negative control RT was positive.

Resulting Cq values and dilution series-based gene specific amplification efficiencies (which were at least 96% for all primer pairs) were entered into the software qbasePLUS (Biogazelle NV, Zwijnaarde, Belgium) for normalization with the two reference genes and subsequent expression analysis. Results were exported as calibrated normalized relative quantity (CNRQ) values. For every gene of interest, the mean CNRQ value of each empty pQE-80L group (one

group per strain) was calculated and used to set all values of the group to 1. CNRQ values of the pQE-80L*::gadX* groups were related to their respective empty pQE-80L expression level (proof of concept: results of experiments at pH 5.0 were related to those at pH 7.0).

2.2.5 *In vitro* adhesion test.

IPEC-J2 cells were seeded at a concentration of 3×10^5 cells per well in 24-well culture plates and grown for two days in DMEM/Ham's F12 medium (Biochrom, Berlin, Germany, pH 7.3) with 5% fetal calf serum (PAA Laboratories GmbH, Cölbe, Germany). Penicillin/streptomycin solution (1%, PAA Laboratories GmbH, Cölbe, Germany) was added for the first day only. Immediately before the adhesion test, IPEC-cells were washed twice with HEPES buffer.

Transformed bacteria were cultivated overnight in LB medium with pH 7.2 containing 50 µg/ml of carbenicillin (PAA Laboratories GmbH, Cölbe, Germany) at 37°C, then diluted in LB medium 1:100 and induced with 0.2 mM IPTG for two hours. Wild type bacteria were treated accordingly but grown without carbenicillin. Then bacterial suspensions were diluted to a concentration of $1x10^8$ cfu per ml DMEM/HAM`s F12 (5 % fetal calf serum, 1% mannose, 0.2 mM IPTG, w or w/o carbenicillin, respectively) and one ml bacterial suspension was given to each well of washed IPEC-J2 cells.

After 3 hours of incubation, IPEC-J2-cells were washed 6 times with PBS to eliminate non-adherent bacteria. For lysis of IPEC-J2 cells, 1 ml of Triton-x-100 (1%) was added. The whole content of each well was transferred to a tube and incubated on ice for 1 h. Quantification of the adherent bacteria was carried out by plating log5-dilution series on LB agar (w/ or w/o carbenicillin; 100 µl/plate) followed by overnight culture at 37°C. Dilutions containing less than 300 CFUs/100 µl were used to calculate the number of adherent bacteria of each well.

2.2.6 Fluorescent-actin staining (FAS) test.

Wild type and transformed porcine EPEC used in the *in vitro* adhesion tests were also examined for their ability to accumulate microfilaments in the apical cytoplasm of IPEC-J2 beneath attached bacteria in a FAS test according to Knutton et al. (1991). The degree of microfilament accumulation in the FAS test is representative of a bacterium's ability to cause attaching and effacing lesions.

IPEC-J2 cells were grown to confluence on 12 mm-cover slips (Hecht-Assistant, Sontheim v.d. Rhön, Germany) and infected with $1x10^7$ cfu of bacteria in 1 ml of DMEM/HAM`s F12 per cover slip. After incubation with the bacterial suspension for 3 h, cells were washed 3 times

with HEPES buffer and incubated for additional 3 hours in fresh DMEM/HAM`s F12. Subsequently, cells were washed three times with PBS-buffer and fixed with 2% paraformaldehyde and permeabilized with 0.005% digitonin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). After 50 µl of a 5 µg/ml fluorescein isothiocyanate (FITC)-phalloidin solution (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) had been added to each well, plates were incubated in the dark for 30 min in a humidified chamber. Then, nuclei and bacterial cells were counterstained with 10 µg/ml of propidium iodide (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for 2 min. Finally, cover slips were mounted on glass slides using Mowiol mounting medium (Clariant, Sulzbach am Taunus, Germany) and evaluated under a fluorescence microscope (DM RB, Leica, Wetzlar, Germany).

2.2.7 pH measurements in the gastrointestinal tract.

In order to determine the physiological pH levels in the different segments of the gastrointestinal tract, digesta samples of stomach, duodenum, mid jejunum, ileum, cecum and mid colon of six healthy pigs were collected from the slaughterhouse. The samples were taken immediately after slaughter and pH levels were determined using a portable pH meter (pH-Meter 1140, Mettler Toledo, Gießen, Germany) calibrated at 37°C with standard buffer (pH 4 and 7) before use. Pigs had been fed a standard fattening diet.

2.2.8 Statistics.

Summarized data of multiple measurements are presented as arithmetic mean and standard error of means (SEM). For statistical analyses Sigma Plot 11.0 (Systat Software Inc., San José, CA, USA) was used. ANOVA or a Student´s t-test was performed and if normality test failed, the Wilcoxon-Mann-Whitney test was applied.

3. Results

3.1 Proof of concept: influence of acidification on GABA production, *gadA/B* and *eae* transcription.

The EPEC wild type strain P185/04-2 produced GABA only at low pH conditions: at pH 5.0 an average GABA concentration of 81.2 ± 10.3 µmol/l was found in the M9 minimum salt medium (Table 3), whereas at pH 7.0 no GABA was detected (detection limit 5 µmol/l). Hence, normalization to the respective OD_{600} values, as performed in all later GABA concentration

measurements, was not feasible and conclusions regarding the rate of increase could not be drawn. However, the increased level of *gadA/B* mRNA was measurable, which was 7.2-fold higher at pH 5.0 compared to pH 7.0 ($p < 0.01$). Growth in acidified medium reduced *eae* transcription by 54% showing a final *eae* mRNA level of 0.46 ± 0.13 compared to the level at pH 7.0 ($p=0.2$).

3.2 Influence of *gadX* overexpression on GABA production.

Two, three and four hours after induction with IPTG, GABA concentrations were measured in the culture supernatant of EPEC strain P185/04-2 transformed with either pQE-80L::*gadX* or the empty vector pQE-80L. Values were normalized to the respective culture density (OD_{600} value). GABA concentrations in cultures of pQE-80L::*gadX* transformants were at least twofold higher than in cultures of empty vector transformants (Table 3). Concentrations determined after two, three and four hours of incubation did not differ from each other significantly. Therefore, GABA concentrations were determined only after two hours for EPEC strains P6414/05-1 and 4181/05-2. GABA concentrations were distinctly lower in these strains, but so were OD_{600} values, hence, after normalization these strains showed GABA differences between empty vector controls and *gadX* transformants comparable to those seen in P185/04-2 with up to 3.4-fold higher values in cultures of pQE-80L::*gadX* transformants.

3.3 Influence of *gadX* overexpression on virulence gene expression

Upon induction with IPTG, *gadX* mRNA levels rose significantly (200 to 1,100-fold) in all *gadX* transformants compared to the respective empty plasmid transformants which were set to 1.0 (Fig. 1, $p < 0.01$). In accordance with these results, transcription levels of *gadA/B* were also markedly higher (14 to 24-fold) in each *gadX* transformant (Fig. 2, $p < 0.05$). To test whether these increased mRNA levels coincided with decreased expression of LEE-encoded virulence genes, transcription of *eae*, *tir*, *cesT* and *escU* were investigated. Interestingly, basal transcription levels of these virulence genes strongly depended on the EPEC strain tested (e.g. only low expression of *eae* in P185/04-2 and almost no *tir* expression in P6414/05-1; data not shown). However, *gadX* overexpression reduced *eae*, *tir*, *escU* and *cesT* transcription in all three strains. This was significant for *eae* in strains P185/04-2 and P6414/05-1 (Fig. 3A), for *tir* in strain P185/04-2 (Fig. 3B) and for *escU* in strain P6414/05-1 (Fig. 3C). As CNRQ data showed large variances between the eight experiments, other values failed to be statistically significant ($p \geq 0.05$).

3.4 Influence of *gadX* overexpression on adhesion ability *in vitro*.

Adhesion and FAS tests were performed to test for the functional consequences of decreased virulence factor expression following *gadX* overexpression. Wild type porcine EPEC and those transformed with empty pQE-80L adhered to IPEC-J2 cells distinctively, whereas bacteria transformed with pQE-80L::*gadX* did not show any adhesion (Fig. 4). Noticeably, not a single colony forming unit was detected to be adherent in any tested EPEC strain after transformation with pQE-80L::*gadX*. The latter finding suggests that *gadX* overexpression leads to functionally relevant downregulation of virulence genes in porcine EPEC.

In agreement with the results of the *in vitro* adhesion test, transformation of porcine EPEC with pQE-80L::*gadX* completely abolished characteristic microfilament accumulation in IPEC-J2 cells that usually occurs upon exposure to LEE-encoding *E. coli* such as EPEC and some EHEC. Thus, adhering bacteria and actin accumulation beneath these bacteria were only found in IPEC-J2 cells cultured with wild type EPEC or their derivatives transformed with empty pQE-80L but not after incubation with pQE-80L::*gadX* transformants (Fig. 5).

3.5 pH measurements in the gastrointestinal tract

As expected, the lowest pH was found in the digesta fluid of the stomach (4.1 ± 0.3, average pH of N=6), whereas the jejunum appeared to have the highest pH (6.5 ± 0.1), decreasing again in the cecum (6.0 ± 0.2, see additional file 1). These findings are very much in line with the results of our previous study (Braun et al., 2015): highest concentrations of GABA were found in the stomach whereas the lowest GABA concentration was detected in the jejunum, increasing again towards the hindgut.

4. Discussion

EPEC have been intensely studied due to their causative role in endemic infant diarrhea in developing countries (Levine & Edelman, 1984; Rowe, 1979; Trabulsi, Keller, & Tardelli Gomes, 2002) and they were even regarded to be the most common bacterial pathogens in infants (Gomes et al., 1991; Katouli, Jaafari, Farhoudi-Moghaddam, & Ketabi, 1990; Khan, Iqbal, Ghafoor, & Burney, 1988; Mubashir et al., 1990). Animals have been considered a significant source for atypical EPEC infections in humans (Chandran & Mazumder, 2013), whereas typical EPEC have mainly been isolated from humans and only infrequently from dogs or pigs (Beaudry, Zhu, Fairbrother, & Harel, 1996; Kaufmann et al., 2006). Thus, EPEC

infections are still of importance, requiring further elucidation of the interplay of EPEC metabolism and the hosts immune system. The fact that crucial functions of pathogenicity – as for the induction of LEE genes by gadX (Branchu et al., 2014)- have also been found to be present in other pathogens like EHEC- might imply a viable approach for new prevention and treatment strategies.

In the present study, we could show that acidification of the growth medium of a wild type EPEC strain can upregulate *gadA/B* expression, lead to measurable concentrations of GABA, and suppress *eae* expression. Plasmid-driven *gadX* overexpression in a pH neutral environment can lead to a complete loss of EPEC's ability to attach to and interact with IPEC-J2 cells due to downregulation of LEE virulence genes. Additionally, *gad*X overexpression in a pH neutral environment also increased GABA production. These results clearly demonstrate that GadX is important for GABA production and a crucial factor of the regulation of AE-promoting EPEC virulence genes.

Regarding Gad expression after acidification, we found a 7.2-fold increased *gadA/B* expression at pH 5.0. Accordingly, we found an average production of 81.2 µmol/l GABA of wild type EPECs at pH 5.0, whereas at pH 7.0 GABA amounts appeared to be below the detection limit of 5 µmol/l. This is not astonishing, as both Gad expression and decarboxylation of glutamic acid are usually stimulated by the presence of excess protons (Castanie-Cornet et al., 1999). Nevertheless, this does not explain why GABA production of the wild type strain at pH 7.0 was below the detection limit, whereas the same strain containing an empty plasmid produced 174-193 µmol/l GABA at pH 7.0. Even though this effect might have been induced by the transformation with the empty plasmid, or by the use of IPTG, we should also consider that the variance between experiments might have been caused by other factors. When looking closer into our data, differences were primarily due to considerable variance in growth performance (expressed as OD_{600}) and baseline expression of the selected LEE genes between strains (e.g., very low expression levels of all factors in strain 4181/05-2), between virulence factors (e.g., generally low expression of *cesT* in all strains) and even between experiments performed under apparently identical experimental conditions.

The importance of growth performance on bacterial gene expression and metabolism is already known from previous studies. For example, Castanie-Cornet et al. (1999) and De Biase et al. (1999) showed that *gadA/B* gene activity depends on whether bacteria are in the log phase or in the stationary phase. Shin et al. (2001) found large variances in virulence gene expression

related to slight changes of growth conditions. Also Yokoigawa, Takikawa, Okubo, & Umesako (2003) showed that growth phase and temperature can influence the expression of Gad genes. Although the experimental setup was always the same, we cannot tell whether the growth phase of all strains and experiments was always identical at the time of sampling. As there was no difference seen between the three time points tested with strain P185/04-2, neither in terms of OD_{600} nor in terms of GABA production, we assumed that after two hours the stationary phase had been reached. Hence, all further experiments (GABA measurements and RNA isolations) were conducted after two hours of incubation. Although GABA concentrations were distinctly lower in strains P6414/05-1 and 4181/05-2, using OD_{600} to normalize GABA levels resulted in GABA differences between empty vector controls and pQE-80L::*gadX* transformants comparable to those seen in P185/04-2 with 2.7 to 3.4-fold higher values.

Our results partially conflict with those of Shin et al. (2001), who tested *gadX*-transformed typical EPEC positive in FAS test despite lower protein expression levels for intimin, Tir, CesT and even slightly decreased *BfpA* expression. They concluded that the production of virulence proteins – although reduced – was still sufficient to induce actin cytoskeletal rearrangements in HeLa cells. Since these authors did not use a vector providing overexpression driven by the *taq* promoter, *gadX* overexpression in transformed EPEC could have been lower compared to our study. This could explain why interaction of transformed EPEC with cultured epithelial cells (HeLa) was not completely abolished in their study.

Dose effects of GadX may also be expected from the results of an analysis of the LEE-encoding EHEC strain O157:H7 (Branchu et al., 2014). This study showed that GadX is not only a negative regulator of LEE1 (thereby suppressing the expression of all other LEE genes) but also a positive regulator of LEE4 and LEE5. For this reason, we focused especially on LEE5 genes (*eae*, *tir*, *cesT*) to evaluate the net effect of *gadX* on these genes. The net effect was consistently negative, although not always statistically significant.

To date, it is well known that GABA - as an exemplary neurotransmitter - has many functions outside the mammalian brain (Erdo & Wolff, 1990). Even for the gut, various effects of GABA have already been described, as for example the alteration of smooth muscle activity (Bayer, Crenner, Aunis, & Angel, 2002) or the activation of antibody secretion by the intestinal epithelium (Jin, Guo, & Houston, 1989). A recent study demonstrated the modulation of abdominal pain by GABA derived from enteric Bifidobateria (Pokusaeva et al., 2016). In accordance with our recent hypothesis that GABA levels influence mucin-1 expression,

McGuckin, Linden, Sutton, & Florin (2011) described that the mucus layer is thickest in the stomach and colon. Apparently this does not only correlate with the number and distribution of mucus-producing cells throughout the gastrointestinal tract but also with our finding that GABA levels are highest in the stomach and colon (Braun et al., 2015). These GABA levels were derived from the same animals and correlate well with measured pH levels. At least for the hindgut, this supports our hypothesis that there is a link between pH and resulting GABA production by enteric bacteria.

Considering these facts, we assume that the GABA content in the intestinal fluid, which might – at least in parts - be derived from bacterial production, can remarkably influence the gastrointestinal physiology via specific GABA receptors. An increase of intestinal GABA production could lead to increased GABA resorption and resulting higher blood levels, however, effects would probably be restricted to the gastrointestinal tract since GABA as a zwitterion cannot pass the blood brain barrier. Thus, effects on the central nervous systems, as they are known from commonly available drugs like GABA receptor agonists or modulators of the GABA synthesis in the brain, are rather unlikely.

However, the interplay between bacterial GABA and the intestine is poorly understood so far. The lack of knowledge in this field offers another interesting subject for further research.

Finally, we have to answer the question of the applicability of our results. The signal that the environment is unfavorably acidic is translated via GadX into reduced efforts of the bacterium to adhere and stay at this unfavorable site as well as into increased efforts to defend (real or fake) intracellular bacterial pH via GABA production. Mimicking such an unfavorable environment by intentional activation of the GadX regulon may reprogram bacteria so that virulence is suppressed. Drugs activating the bacterial GadX regulon are not available so far but there might be other options to modulate this pathway. For instance, Branchu et al. (2014) postulate that the nitric oxide (NO) sensor nitrite-sensitive repressor (NsrR) is a direct positive regulator of the transcription of LEE1, LEE4 and LEE5 genes and an indirect repressor of *gadE* and *gadX* genes. However, supporting antibiotics in their mode of action or even replacing them by GadX modulators is still a long way off and requires further research.

5. Conclusions

We conclude that intensified *gadX* activation in EPEC can abolish EPEC´s ability to adhere to the intestinal epithelium by reducing expression of virulence genes like *intimin*. Since production of GABA, a neurotransmitter with potentially positive effects on the mucosal barrier, was increased at the same time, the GadX regulon could be an interesting target for the prevention of EPEC-associated postweaning diarrhea in piglets.

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8. Declarations

The authors declare no conflict of interest related to this work.

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Authors' contributions: JRA and CD conceived and designed the experiments. HSB, GS, KK and CD performed the laboratory work. KK and RB acquired and provided EPEC isolates. GS performed the transformation of the EPEC isolates. HSB, JRA, KK, RB, and CD analyzed the data. HSB, JRA and CD prepared the manuscript. All authors participated in interpretation and discussion of results and have read and approved the final manuscript.

9. References

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10. Figures

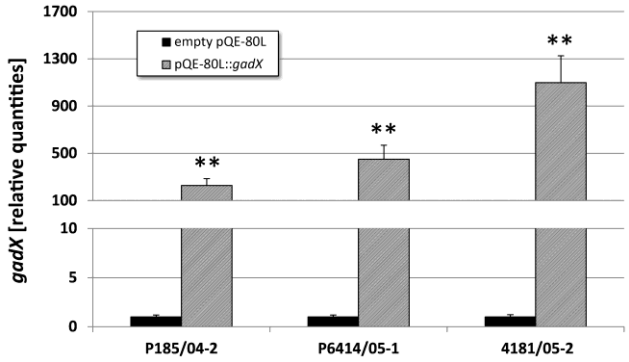

Figure 1. Transcription of *gadX* in transformed EPEC strains.

Plasmid (pQE-80L)-encoded *gadX* transcription was induced in two atypical (P185/04-2 and 4181/05-2) and one typical EPEC strain (6414/05-1) with IPTG for two hours. Analysis by RT-qPCR revealed that *gadX* mRNA was significantly increased in *gadX* transformants (hatched bars) compared with respective transformants harboring the empty plasmid pQE-80L only (black bars, mRNA level set to 1.0). Data are given as arithmetic means and standard errors of means of eight replicates (duplicates tested on four consecutive days); **p < 0.01

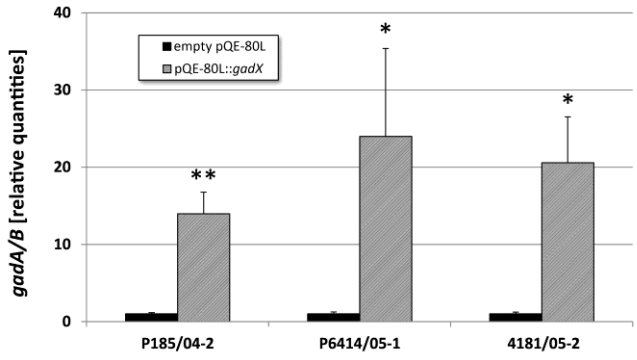

Figure 2. Transcription of *gadA/B* in transformed EPEC strains.

Upon IPTG induction, those transformants harboring pQE-80L::*gadX* showed significantly higher *gadA/B* transcription (hatched bars) than the respective transformants harboring the empty plasmid pQE-80L only (black bars, mRNA level set to 1.0). Data are given as arithmetic means and standard errors of means of eight replicates (duplicates tested on four consecutive days); **p < 0.01, *p < 0.05

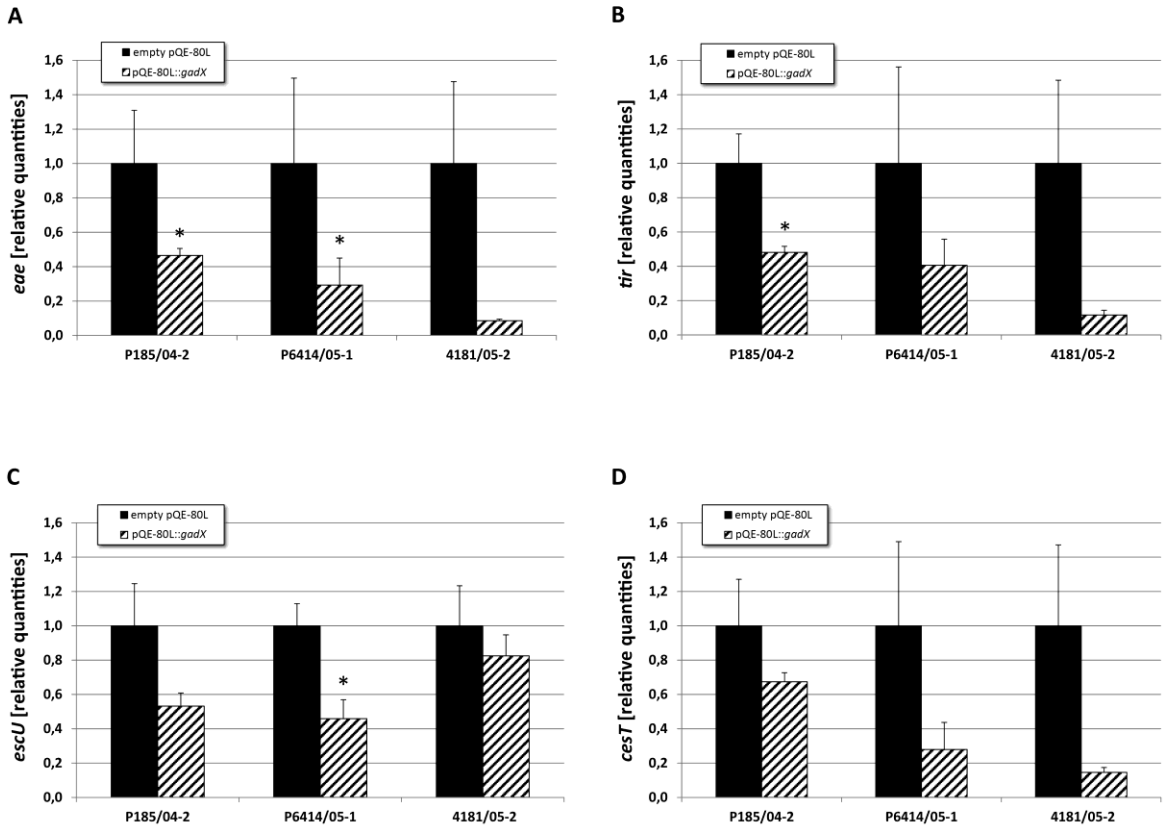

Figure 3. Transcription of virulence genes in transformed EPEC strains.

Transcriptions of *eae* (A), *tir* (B), *escU* (C) and *cesT* (D) in IPTG-treated pQE-80L::*gadX* transformants (hatched bars) were compared to those of the respective transformants harboring the empty plasmid pQE-80L only (black bars, mRNA level set to 1.0). Despite a high variability in baseline expression (data not shown), all virulence factors were numerically or significantly down-regulated in all EPEC transformants that had shown *gadX* overexpression (see Fig. 1). Data are given as arithmetic means and standard errors of means of eight replicates (duplicates tested on four consecutive days); *p < 0.05

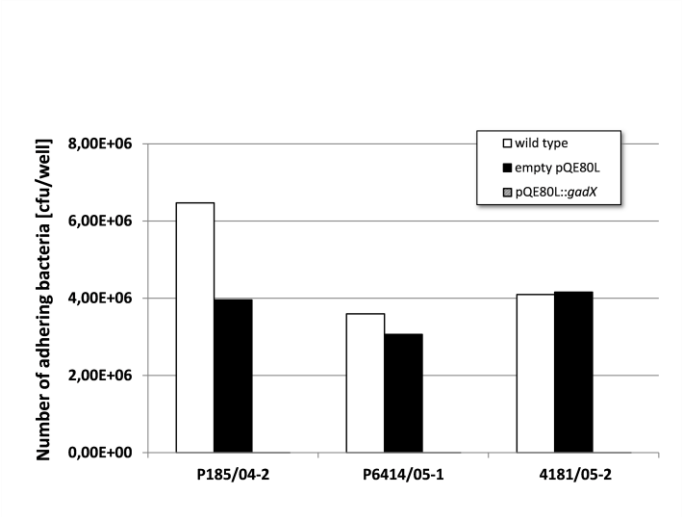

Figure 4. Adhesion of wild type and transformed EPEC strains to IPEC-J2 cells.

After transformation with pQE-80L::*gadX*, all three strains lost their ability to adhere to jejunal porcine epithelial cells (IPEC-J2) *in vitro* (hatched bars; not visible as the number of adhering bacteria was below the detection limit of the assay which was 10 cfu/well). In contrast, all wild type strains were able to adhere, as well as the respective transformants harboring empty pQE-80L. (one replicate per strain)

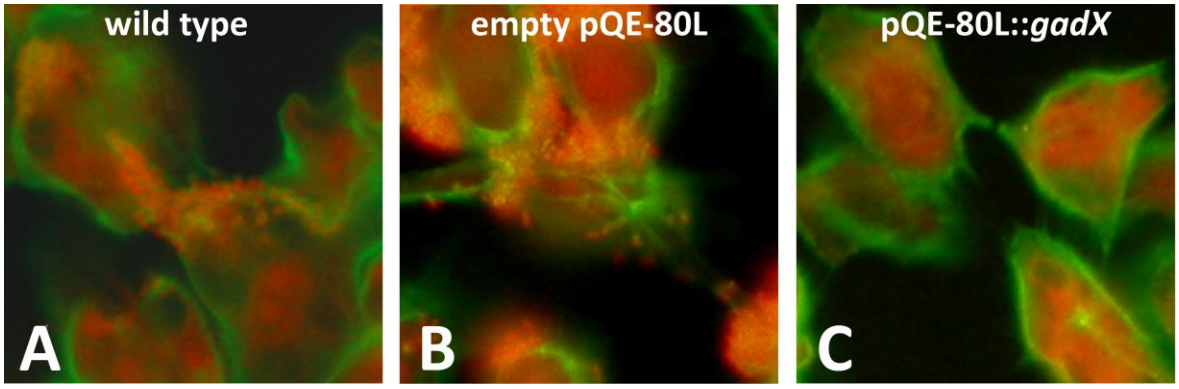

Figure 5. IPEC-J2 cells in the fluorescent-actin staining (FAS) test of EPEC strain 4181/05-2 and its transformants.

In an IPTG-containing medium, IPEC-J2 cells (red nuclei, green actin/cell membranes) had been incubated with the (A) wild type strain, (B) pQE-80L transformant, or (C) pQE-80L::*gadX* transformant. Localized adherence of bacteria (conglomerates of tiny red spots) and accumulated actin (green accumulations right underneath bacteria) were only visible in A and B. Original magnifications, × 1,000.

11. Tables

Table 1. Main characteristics of the used EPEC strains (isolated from diarrheic pigs).

Strain designation	Serotype	*eae* type	*bfpA* plasmid	FAS intensity[1]
P185/04-2	O76:H7	g2/q	No	1
P6414/05-1	O40:H10	e1	Yes	2
4181/05-2	Ont:H-	k	No	2

[1]FAS intensity was scored from 0 to 3 (0 = 0% of all cells positive, 1: < 30 % of all cells positive; 2: 30 - 60 % of cells positive; 3: > 60 % of cells positive)

Table 2. Sequences (5′ to 3′) of primers used for RT-qPCR and resulting amplicon sizes.

Gene	Forward primer	Reverse primer	Amplicon Size [bp]
gadX	ATGACGCCCACAGAGTATCAG	GGTCAGTGCCGTAAAAATTCCC	101
gadA/B	TTACCAGGTTGCCGCTTATC	ACGCAGACGTTCAGAGAGGT	163
eae	ACCGTCATATCCGGCATTAG	ACCATGACGGTAATCGATCC	169
tir	TCAGGGGAGAAAACAATGAACG	GCCACTACCTTCACAAACAGAC	101
cesT	CGACAGATAACCCTAACTTCGC	CCATCGACTTAACGACGACTTC	186
escU	GGGCGATGTAACAAAAAGTGA	CCGCACCTAATATTTCACGA	176
cysG	TTTCTGGGAGAAATTGTTCG	TTGCAGTCCTTTCAGTGTCA	181
gapdh	TCCGTGCTGCTCAGAAACG	CACTTTCTTCGCACCAGCG	299

Table 3. Normalized GABA concentrations in culture supernatants of EPEC.

Strain	Plasmid	Hours of IPTG induction	GABA* [µmol/l]	Mean OD_{600}	Normalized GABA* [µmol/l	Significance **
P185/04-2	Wild type, pH 7.0	-	< 5.0	0.540	-	
	Wild type, pH 5.0	-	81.2 ± 10.3	0.524	156.5 ± 26.2	a
	Empty pQE-80L	2	181.1 ± 13.1	0.659	274.7± 19.6	b
		3	192.8 ±	0.665	290.7 ± 63.9	a, b
		4	173.5 ±	0.674	260.5 ± 65.5	ab
	pQE-80L::*gadX*	2	398.2 ±	0.634	628.3 ± 28.1	c
		3	417.3 ±	0.669	624.1 ± 25.5	c
		4	366.6 ±	0.681	537.2 ± 58.7	c
P6414/05-1	Empty pQE-80L	2	16.5 ± 0.7	0.308	53.7 ± 3.7	d
	pQE-80L::*gadX*	2	53.2 ± 5.4	0.363	146.8 ± 13.8	a, e
4181/05-2	Empty pQE-80L	2	19.6 ± 1.6	0.408	48.3 ± 5.0	d
	pQE-80L::*gadX*	2	67.7 ± 5.5	0.413	164.4 ± 13.9	a, e***

* data are given as arithmetic means and standard error of the mean of three replicates (except for wild type P185/04-2 where four replicates where used)

** supernatants that do not share the same letter, differ significantly ($p < 0.05$)

*** if normality test failed, a Wilcoxon-Mann-Whitney test was performed instead of Student´s t-test

11. Supplementary

Additional file 1

Table 1 Suppl.: pH levels of the digesta in different gastrointestinal segments of six pigs

Segment	pH level							
	pig 1	pig 2	pig 3	pig 4	pig 5	pig 6	**mean**	SEM
Stomach	5.0	4.1	3.93	3.85	2.86	4.9	**4.11**	0.32
Duodenum	6.3	5.5	4.07	6.3	6.48	5.41	**5.68**	0.37
Jejunum	6.2	6.86	6.22	6.24	6.88	6.38	**6.46**	0.13
Ileum	6.5	6.43	6.58	5.81	6.5	6.2	**6.34**	0.12
Cecum	6.05	6.4	5.75	5.38	5.88	6.25	**5.95**	0.15
Colon	6.2	6.4	5.98	5.75	6.37	7.07	**6.30**	0.18

Measurements were performed immediately after slaughtering using a portable pH meter; Data are given as arithmetic means and standard error of the mean (SEM)

6. General Discussion and Conclusions

This work sought to evaluate whether GABA-dependent interaction between the intestinal epithelium and EPEC could be a relevant target for alternative approaches to combat diarrhoea. Although the experiments were conducted to elucidate the particular role of GABA and EPEC in pigs, the general findings of this work might have relevance for other species as well. Therefore, the results of the studies will be discussed in a translational context.

Diarrhoeic diseases are still among the most prevalent health issues world-wide, posing in particular a serious risk to children and elderly people. Also farm animals are regularly affected by outbreaks of severe diarrhoea (Thumbi, Njenga et al. 2015). The PWD of piglets is one important example as has been presented in Chapter 2.5.1.The occurrence of bacterially induced diarrhoea in animals does not only cause ethical and economic problems but is also a threat to human health as many enteric pathogens are zoonotic (Filippitzi, Goumperis et al. 2017, Manyi-Loh, Mamphweli et al. 2018). Strategies to prevent such diseases are challenging because animals of various species provide a habitat for bacterial pathogens that is hard to control. The increasing resistance of pathogens to antibiotics, which is discussed to be linked to the excessive use of antibiotics, impedes the suppression of bacterially induced diarrhoea even more: In this regard, it is particularly alarming that bacteria are able to transfer resistance genes among each other, which enables antibiotic resistance to spread rapidly (Barlow 2009). This is one of the major reasons why most countries, including those in the European Union, have recently restricted or banned in-feed antibiotics (Anadon 2006). This, in turn, means that ground-breaking new solutions are required to combat diarrhoea and other bacterial induced diseases in livestock and humans. So far, effective alternatives for antibiotics are still lacking but recent advancements in research offer various promising approaches which might contribute to new solutions.

The aim of the present study was to elucidate whether *gadX* stimulation leads to an increased GABA production and repressed virulence in EPEC and improves defence mechanisms of the gut at the same time. In the first part of this work, described in Chapter 4, the impact of GABA on porcine intestinal epithelium was studied in an *ex vivo* experiment. It turned out that GABA selectively increases the expression of mucin-1 (MUC1), suggesting a beneficial effect for epithelial protection. In the second experiment, presented in Chapter 5, it was shown that the

stimulation of the transcription factor GadX in EPEC - either by an acidic pH or by an induced overexpression in *gadX*-transformed strains - leads to a decreased expression of virulence factors and a significantly reduced ability to attach and adhere to intestinal epithelial cells *in vitro*.

In combination, these results could open a view on new solutions in the treatment of diarrhoea. However, the relevance of these results and their translatability into potential future prevention and treatment approaches have to be discussed thoroughly.

GABA is Naturally Found in the Digesta

Although GABA is a non-proteinogenic amino acid, it belongs to the regular diet as it is a significant component of the free amino acid pool of all kinds of plants and also available in dairy products. This may be due to the fact that the ability to convert glutamate into GABA and CO_2 by use of the enzyme glutamic acid decarboxylase is widely distributed throughout the animal and plant kingdom – from chicken and pig (Gottlieb, Chang et al. 1986, Bao, Cheung et al. 1995) down to mung bean (Kulkarni and Sohonie 1956) and barley (Inatomi and Slaughter 1975).

Depending on the diet, GABA levels in the feed/food differ a lot; e.g. in grass silages, GABA concentrations range between 0.07 and 12 g/kg dry matter, whereas fresh grass can contain up to 13 g/kg dry matter (Bond, Powell et al. 1984, Dawson and Mayne 1996, Coenen, Scholz et al. 2015). In certain black teas for human consumption, GABA content is around 2.3 g/kg (Zhao, Li et al. 2015) and in germinated brown rice it was shown to reach up to 100 mg/kg (Wu, Yang et al. 2013).

Nevertheless, the amount of GABA in the digesta depends not only on the diet but also on the location within the gastrointestinal segment. In the present study (Chapter 4), GABA levels of 27.6 µmol/l on average and maximum levels up to 100 µmol/l were found in the pig´s stomach. In the colon, GABA levels were 21.9. µmol/l on average with values of up to 53.2 µmol/l, which is in line with previous findings in other species (Wright and Hungate 1967, Pokusaeva, Johnson et al. 2017). GABA levels found in the jejunum were only 3.9 µmol/l on average (maximum was 26.2 µmol/l) suggesting that the physiological GABA concentration in the jejunum is rather low compared to other gastrointestinal compartments. It seems reasonable that GABA in the stomach and small intestine is derived from different sources than that of the large

intestine. The increase of the GABA concentration from the ileum towards the hindgut could be a result of water resorption but could also imply a source of GABA other than the diet.

It is well known that GABA is produced not only by EPEC but also by several enterobacteria such as *Lactobacilli* and *Bifidobacteria* (Bravo, Forsythe et al. 2011, Yunes, Poluektova et al. 2016). Thus, the GABA content of the large intestine could be of bacterial origin. The fact that the large intestine is the primary site for the microbial colonization (Hao and Lee 2004) provides a reasonable explanation for the finding that GABA levels are significantly higher in the large intestine compared to the small intestine. Yunes et al. analysed 135 strains of human gut-derived *Lactobacilli* and *Bifidobacteria* for their ability to produce GABA. Fifty-eight strains were able to produce GABA, some of them produced even up to 6 g/l culture medium (Yunes, Poluektova et al. 2016).

Auteri et al. observed that 10 to 50 µmol/l of GABA potentiated colonic peristalsis while high concentrations (500 µmol/l to 1 mmol/l) inhibited motility (Auteri, Zizzo et al. 2014). In the present *ex vivo* study, 50 µmol/l and 1 mmol/l GABA were applied to jejunal mucosa. Both concentrations led to a significantly increased MUC1 expression compared to the control group. Hence, a concentration of 50 µmol/l might already exceed the physiological GABA level of the jejunal digesta by far thereby causing the observed effect. Unfortunately, in this study it was not evaluated whether the observed effect was due to the high concentration of GABA or the complete lack of GABA in the control group. Other studies are also lacking this comparison. However, since the average GABA concentration in the jejunal digesta were below 5 µmol/l (the lowest concentration found was 1.8 µmol/l), it might be reasonable to conclude that the control group without GABA is comparable to a physiological set up. Nevertheless, to corroborate the hypothesis, more concentrations (especially at low levels) should be tested.

Effects of GABA in the Gut

It is beyond dispute that GABA affects intestinal motility and secretion but there is also evidence for an involvement of GABA in neuroimmune reactions (Auteri, Zizzo et al. 2014). It is not clear, however, what mode of action is leading to these various effects. Several possibilities are discussed, among which a major involvement of the CNS and the vagus nerve seems to be likely. Nevertheless, many experiments demonstrating an impact of GABA on immunological and secretory functions were conducted *in vitro* or *ex vivo* where central or even vagal afferents can play nor or an only minor role. Consequently, there must also be a direct effect on the epithelium.

In the present study (Chapter 4), the effect of GABA and glutamine application on porcine jejunal epithelium was evaluated in an Ussing chamber approach. The results showed that GABA significantly increased MUC1 expression on mRNA and protein levels. Furthermore, application of the GABA precursor glutamine showed the same tendency. Hence, it can be concluded that the frequently reported beneficial effects of glutamine on the gut could partially be evoked by its role in the metabolism of GABA. In the gut, mucins, especially MUC1, are believed to be an important factor of the immune response to infections. Besides a significant contribution of MUC1 to the mucosal barrier, it is also discussed to be involved in the regulation of T helper cell responses, the control of inflammatory processes and the response to bacterial invaders (McAuley, Linden et al. 2007, McGuckin, Every et al. 2007, Nishida, Lau et al. 2012). It is tempting to speculate that a recent study of Mao et al. could support the hypothesis that GABA directly affects the MUC1 expression of jejunum epithelial cells: in this experiment, weaned piglets supplemented with the probiotic strain *L. rhamnosus* revealed enhanced mucosal barrier functions including increased MUC1 expression (Mao, Gu et al. 2016). *L. rhamnosus* is known for its abundant GABA production, e. g. it is discussed to be one of the potential sources of GABA in the large intestine (Franciosi, Carafa et al. 2015).

The results of the presented *ex vivo* experiments with isolated epithelium justify the conclusion, that GABA can act directly on the epithelial cell layer. Not much is known about the mode of action of GABA in the intestinal epithelium. As $GABA_B$ receptor expression by the epithelium was demonstrated in the present experiments (Chapter 4), it seems likely that GABA acts directly via those receptors. Furthermore, the detected mRNA expression levels of $GABA_B$ receptors were strikingly constant in all mucosal tissue samples analysed, implying a highly consistent expression pattern of the epithelial cells. If cell types such as enterochromaffin cells (which were certainly included in the mucosal samples) would be the main source of $GABA_B$ receptors mRNA, one would expect rather inhomogeneous expression levels between the samples which were obtained from different sites of the jejunum. Hence, it seems reasonable that $GABA_B$ receptors are indeed expressed by epithelial cells. Own unpublished results revealed, that even IPEC-J2 cells show a significant and homogenous expression of $GABA_B$ receptor mRNA, thereby supporting the theory that GABA may have indeed a significant and direct function on the epithelial cell itself. Other authors found expression of $GABA_A$ receptors in the jejunal epithelium of rats (Li, Xiang et al. 2012). Comparable findings have been reported in studies evaluating the significance of the GABA metabolism in airway epithelium, contributing to the hypothesis that epithelial cells are directly affected by GABA (Xiang, Wang

et al. 2007). In the airways, GABA is most likely derived from the epithelial cells as they have been proven to strongly express GAD65 and GAD67 (Gallos, Townsend et al. 2013).

In contrast, in the present study (Chapter 4), only a weak expression of GAD65 and no expression of GAD67 were found in the jejunal epithelium, suggesting that GABA in the digesta might not be derived from the epithelium but rather from an external source such as diet or microbiota. Nevertheless, the response to GABA, independent of its origin, shows similarity in intestinal and airway epithelium as there is also evidence for a correlation of GABA and mucin amounts. In fact, $GABA_A$ receptor antagonists have been shown to reduce mucin production of goblet cells (Xiang, Wang et al. 2007) and the expression of GAD67 in the airway epithelium of healthy smokers was correlated with mucin 5A expression (Wang, Wang et al. 2010). It can, hence, be assumed that extracellular GABA binds to the GABA receptor of the epithelial cell. How this signal is then converted into the induction of increased mucin expression is not known yet and requires further research.

The validity of *in vitro* experiments and their *in vivo* transferability depends on the concentration levels applied. In view of the fact that physiological GABA levels in the stomach and in the large intestine can become impressively high, whereas GABA concentrations in the jejunum are rather moderate, the relevance of many *in vitro* studies should be discussed cautiously. At least it has to be considered to what extent topically applied concentrations of GABA are related to the physiological levels and whether such concentrations would be feasible in the targeted gastrointestinal segment of an *in vivo* set up.

Taking together, the above discussed studies regarding the GABA receptor expression in the mucosa and the data of the present study lead to the conclusion that there may be a direct effect of GABA on the jejunal epithelium. However, it should be kept in mind that in *ex vivo* or *in vitro* approaches potential vagal effects cannot be evaluated. Since their significance for GABA`s beneficial effects *in vivo* is undeniable, potential central and vagal effects will be discussed below.

Effects of GABA after Resorption

In the present study, GABA levels in the pig feed were approximately 180 µmol/l. The broad range of GABA content in the diet could explain the highly different GABA levels observed in the stomach. The decreasing GABA levels from the stomach towards the beginning of the large intestine are most certainly due to absorption processes in the small intestine. It is a matter of

debate whether this absorption occurs mainly paracellularly via passive diffusion or transcellularly via specific transporters. Rackwitz and Gäbel recently postulated that the major part of GABA from the digesta in the ovine jejunum is taken up paracellularly (Rackwitz and Gäbel 2017). In their *ex vivo* experiments, they found no evidence for any participation of active transport processes. In contrast, other authors believe in an involvement of the putative amino acid transporter 1 (PAT1), which is expressed in the small intestine of rats and humans (Thwaites and Anderson 2007). In the experiments of Rackwitz and Gäbel, however, neither β-alanine, glycine nor changes in the pH had an impact on GABA transport from the mucosal to the serosal side, suggesting that GABA does not use the same transport protein as other zwitterionic amino acids. However, GABA concentrations used in the study by Rackwitz and Gäbel (1-50 mmol/) were markedly higher than the GABA levels that were measured in the jejunal digesta in the present study. Hence, the unphysiologically high levels of GABA used by Rackwitz and Gäbel might have masked active transport processes. Furthermore, Rackwitz and Gäbel calculated a constant absorption rate of 20 nmol/cm^2/h in the ovine jejunum (adding up to 7 mmol/day or 0.7 g/day per sheep, which appears rather low compared to the potentially high GABA amounts in the diet), raising the question about the fate of the remaining GABA in the diet. Interestingly, Nácher et al. demonstrated strong evidence for the involvement of both active and passive transport processes in an *in situ* study of the small intestine of rats (Nacher, Polache et al. 1994). In that experiment, GABA absorption followed Michaelis-Menten kinetics and was also influenced by the presence of β-alanine. In the opinion of Nácher et al., the partially competitive uptake of β-alanine and GABA provides evidence for a commonly used transport protein such as PAT1. Moreover, the authors observed a saturation process in the active transport that was accompanied by an increase in passive transport. In view of these facts, it can be concluded that the major part of GABA from the diet is taken up in the small intestine and absorption apparently reacts to changes in GABA concentration of the diet by adaptation of passive transport processes.

The hypothesis that intestinally absorbed GABA leads to elevated plasma levels of GABA which affect the CNS, is commonly used for the marketing of various GABA-rich nutritional products (such as rice or tea). Marketing advertisements promise anxiolytic effects, promotion of sleep or pain reduction. So far, it has not been proven that GABA as a zwitterion can cross the blood-brain barrier even though a few authors found evidence suggesting that this possibility should not be excluded (Boonstra, de Kleijn et al. 2015). However, even if GABA would be able to permeate the blood-brain barrier, it is questionable whether the amount of

GABA derived from such supplements would be sufficient to reach adequate concentrations in the brain to induce any biologically relevant effect. Hence, it is rather unlikely that orally administered GABA can lead to predominantly $GABA_A$ receptor-mediated effects in the CNS although it is beyond dispute that GABA plays a central role in the pathophysiology of many neuropsychiatric disorders.

It is known that anxiety disorders, addiction, depression or even epilepsy are often related to a dysfunction of the GABA metabolism in the CNS. Therefore, $GABA_A$ and $GABA_B$ receptor agonists/antagonists as well as compounds interacting with GABA transporters are in the focus of the development of potential therapeutics, some of which are available on the market since many years.

Compared to that, only few studies have elucidated the relation of peripheral GABA and its impact on neuropsychiatric disorders of the CNS. The role of plasma GABA levels in the pathogenesis of such diseases is a matter of debate. Physiological plasma levels depend on the species; e.g. in healthy children, plasma levels were found around 14 ng/ml, whereas in dogs, average plasma levels have been reported to be approx. 20 mg/ml (which appears high and has not been verified) (Thompson, Schafer et al. 1985, Prosser, Hughes et al. 1997), but there is also a great deal of variance between individuals: Knych et al. analysed 147 horses of different breeds for their plasma GABA levels and found an age and breed independent range of 10 to 64 ng/ml (Knych, Steinmetz et al. 2015), indicating strong individual differences.

Nevertheless, it has been reported that plasma GABA levels of patients with mood disorders are often lower than those of healthy subjects (Petty, Kramer et al. 1990). Some authors suggest that there is a genetic predisposition for low GABA levels, both in the plasma and in the brain, which is accompanied by an increased susceptibility for mood disorders (Petty 1995, Petty, Fulton et al. 1999). On the other hand, in young autistics, plasma levels of GABA seem to be elevated (Dhossche, Applegate et al. 2002). It is assumed that the imbalance in the GABAergic system in patients suffering from autism is a consequence of neuroinflammation (El-Ansary and Al-Ayadhi 2014).

In view of the fact that neurotransmitter concentrations in the brain are highly regulated and well-controlled, and that severe neuropsychiatric disorders can occur in the absence of a tight regulation, it seems implausible that the highly variable GABA intake from the diet could be directly linked to the respective levels in the CNS, which would pose a risk to interfere with the fine-tuned, cerebral GABA balance. Moreover, Knych et al. have shown that the GABA plasma

level of horses did not change after oral administration of a solution containing 1.65 g GABA (Knych, Steinmetz et al. 2015). Other reliable studies, which prove the dependence of GABA blood levels on GABA uptake from the food/feed are lacking. Nevertheless, as described above, it is beyond dispute that the major proportion of food-derived GABA is taken up into the blood; however, GABA then follows the fate of most other amino acid derivatives in the blood: it is taken up by the liver where degradation starts with the enzyme ABAT (Tillakaratne, Medina-Kauwe et al. 1995). Hence, excess food-derived GABA uptake is supposed to lead to a higher activity of hepatic GABA degrading enzymes in order to stabilize the GABA blood level. Therefore, also bacterially derived GABA most likely is taken up into the blood and degraded in the liver, but prior to this it might interfere directly with the epithelium and/or the vagal system (as described in Chapter 2.4.3).

There is a growing body of evidence that GABA is involved in the complex communication processes of the gut-brain axis. Bravo et al. postulated that the positive effects of the probiotic strain *L. rhamnosus* are caused by changes in the GABAergic system (Bravo, Forsythe et al. 2011). They found evidence for $GABA_A$ and $GABA_B$ receptor mRNA changes in the brain after oral administration of the bacterium to mice and claimed that *L. rhamnosus* induces such changes by affecting the vagus nerve in the ENS. In their opinion, the vagus nerve itself subsequently leads to a modified release of GABA in the mouse brain, which can cause changes in behaviour and mood. How the bacterium exactly stimulates the vagus nerve is not clear but Bravo et al. could show that *L. rhamnosus* influenced physiological and behavioural responses to stress as well as GABA receptor expression in the brain, whereas vagotomised mice showed significantly different reactions.
In a recent study by Pokusaeva et al. the GABA-producing *Bifidobacterium dentium* was found to modulate visceral pain, supporting the hypothesis that bacteria-derived GABA could interact with the vagal system (Pokusaeva, Johnson et al. 2017).

Since many studies - including own unpublished results - demonstrated a broad distribution of GABA receptors in various parts of the gut (from epithelial cells to enteric neurons of the submucosal and myenteric plexus, interneurons and also mucosal endocrine-like cells), it is tempting to speculate that besides affecting epithelial functions directly, GABA might play a central role in the gut-brain axis by communicating with the vagus nerve.

Stimulation of Bacterial GABA Production May Inhibit Bacterial Invasion

Consistent with the findings of the present study, most of the authors agree that GadX is a central target in EPEC pathogenicity. Thus, the remainder of the discussion will focus on the question how bacteria-derived GABA could affect gut health and which approaches could be promising to induce GABA production in EPEC.

Compared to the production capacities of *L. rhamnosus*, the ability of *E. coli* to produce GABA appears only marginal, even though some genetically engineered strains have recently gained interest for their highly efficient GABA production (up to 1 g/l culture medium) for industrial applications (Dung Pham, Somasundaram et al. 2016, Pham, Somasundaram et al. 2016). In accordance to Yunes et al. (Yunes, Poluektova et al. 2016) who found significant differences between *Lactobacilli* strains, differences between the evaluated EPEC strains also occurred in the present study (Chapter 5). After acidic induction of GadX in one wild type strain, 81.2 µmol of GABA per litre of culture medium were measured. At pH 7, GABA levels were below the detection limit of 5 µmol/l, indicating that at a pH level around 6.4, which is considered physiological in the jejunum, GABA production is usually weak. However, in other strains which had been transformed with an empty plasmid, GABA production at pH 7 ranged between 17 and 180 µmol/l depending on the strain. The same strains harbouring the inducible *gadX* plasmid reached GABA levels between 50 and nearly 400 µmol/l (Chapter 5). Even though the production capacity of the *gadX*-transformed EPEC might be artificially enhanced, the analysed GABA levels where still comparable to those produced by wild type isolates.

Compared to the EPEC strains of the present study (Chapter 5), the GABA production ability of *L. rhamnosus* is assumed to be higher. Lin reported GABA production of 0.5 to 20 mmol/l in 24 hours depending on the growth conditions (Lin 2013). However, due to potential differences in growth state and also growth conditions, the amount produced by *L. rhamnosus* in their study is not directly comparable with the amount of GABA produced by the EPEC strains in the present study. Additionally, it has to be considered that probiotic bacteria like *L. rhamnosus* colonize preferably the colon whereas EPEC favour the jejunal mucosa (Bardiau, Szalo et al. 2010), where small changes in GABA concentration could have a great impact.

Besides influencing the host, the *gadX*-driven GABA accumulation in the jejunum could have another beneficial effect: inhibition of bacterial invasion. Not much is known about the direct effect of GABA on bacteria; however, there is evidence that at least some bacterial species are affected by high GABA levels. Accumulation of GABA in plants can inhibit bacterial invasion. Several plants have been observed to increase GABA levels in wounded tissues, which seems

to reduce the virulence of certain *Agrobacteria* strains (Shelp, Bown et al. 2006, Petrivalsky, Brauner et al. 2007). Chevrot et al. proposed that GABA modulates a quorum sensing signal in the bacterium by inactivating homoserine lactone. Furthermore, GAD-overexpressing transgenic tobacco plants were less susceptible to the pathogen *Agrobacterium tumefaciens* (Chevrot, Rosen et al. 2006). Recent studies suggest that bacteria such as *Agrobacteria*, *Rhizobia* and also *Pseudomonas* can harbour a GABA-binding protein (Planamente, Mondy et al. 2012). However, in contrast to the inhibiting effect of GABA on *Agrobacteria*, the exposure of 10 µM of GABA to *Pseudomonas aeruginosa* increased cytotoxicity and virulence while growth kinetics or motility was not modified (Dagorn, Hillion et al. 2013).

The hypothesis that stimulation of the transcription factor GadX leads to reduced virulence of EPEC strains has been evaluated in various approaches. In the own experiments described in Chapter 5, GadX expression was stimulated via either acidification or an inducible GadX-expressing plasmid. In both trials, GABA production and *gadA/B* expression were significantly increased while mRNA expression of the key virulence gene intimin was reduced. In the experiment with the *gadX*-transformants, the expression of further genes of LEE was also reduced upon *gadX* induction although the difference was not always significant. The lack of significance for the latter finding might have been caused by differences in growth phase or might be strain-specific as two strains showed weak expression of virulence genes in general (even without *gadX* induction).

There is also evidence for other non-LEE encoded regulators of LEE, which are either part of a superordinate or at least an equivalent virulence regulation system (Franzin and Sircili 2015). This could explain why the basal expression of LEE genes was differing substantially between strains already in absence of *gadX* induction.

However, several other factors can affect bacterial gene expression. For instance, the transcription factor H-NS, a DNA binding protein and global regulator, is known to be a potent modulator of LEE gene expression. H-NS, in turn, is affected by environmental factors such as osmolarity, temperature and nutrient status (Williams and Rimsky 1997). Another transcription factor superiorly involved in LEE regulation is the alternative sigma factor 54 (RpoN). This factor was reported to regulate acid resistance in EPEC (including the Gad system) and virulence gene expression of LEE (Riordan, Tietjen et al. 2010). Induction of RpoN – and thereby enhanced LEE gene expression - is not only influenced by environmental factors such as nitrogen assimilation in the growth medium but also by the altering growth phase. Hence, it

is not deniable that EPEC virulence can also be regulated by genes of superior hierarchy, which might strongly affect *gadX* expression.
Certainly, those factors could have influenced virulence expression in the experimental set ups of the present study.

For bacteria, it seems beneficial to reduce virulence and thereby their ability to adhere if the environment is unfavourable. Hence, virulence gene expression can be reduced either when the bacteria enter a certain growth phase (indicating a high population density, which might be reached relatively fast in an overnight culture, depending on the strain) or when nutrient supply, temperature or pH milieu is suboptimal. In contrast to the variable results of the virulence mRNA expression observed in the study of Chapter 5, the *in vitro* adhesion ability of all tested EPEC strains carrying an empty plasmid was high as expected, while *gadX* induction completely abolished the adhesion ability.
Due to the different experimental set ups in the *in vitro* adhesion test and the qPCR experiments, the environmental conditions for the bacteria were fairly different. Hence, environmental factors which activate superior transcription factors that - in turn - increase *gadX* expression, might have been abolished in the adhesion test approach and in the FAS test. Consequently, it can be concluded that GadX plays indeed a role in EPEC virulence gene expression, although there might be other factors that can interact with LEE.

Future Opportunities and Outlook

In summary, there is evidence that stimulation of GABA production in EPEC via the GadX-Gad A/B axis could have beneficial effects for the small intestine in terms of epithelial immunological functions (e.g. mucin production) but might also affect vagal functions with consequences for motility and secretion. These effects might differ between species but it can be assumed that the general theory is at least in parts applicable to most species.

Furthermore, it is generally accepted that increased GadX expression can contribute to a reduced virulence of EPEC although there might be other potential targets. The interesting question, however, is how GadX stimulation could be achieved and whether it could be relevant for future *in vivo* approaches.

In order to activate *gadX e*xpression in EPEC, two approaches seem to be promising:

- GadX stimulation by a targeted pH reduction in the jejunum or
- use of compounds that activate *gadX* expression directly.

Modulation of the intestinal pH, especially in the jejunum, is an ambitious goal that many researchers have targeted since a low gastrointestinal pH is generally considered to inhibit microbial colonization (von Rosenvinge, O'May et al. 2013). There are few substances, e.g. carbonic anhydrase inhibitors such as acetazolamide, which interfere with the bicarbonate metabolism (Phillips and Schmalz 1970). Furthermore, mannose and fructose have been shown to increase hydrogen concentration (Turnberg, Fordtran et al. 1970). Unfortunately, none of these approaches have been successful in consistently reducing the jejunal pH. Compounds directly interfering with the vagal system to increase acid production might be strikingly effective but bear the risk of strong side effects that outweigh the benefits of such an approach.

Especially in broiler chickens, several attempts have been made to reduce pH levels in the small intestine by feeding diets high in fibre, essential oils or bioactive plant compounds like for example the leaf meal of *Moringa oleifera*. Even though slight effects (such as modifications of acetic and propionic acid concentrations) were observed in some studies, the impact on the pH was not consistent (Kalmendal, Elwinger et al. 2011, Nkukwana, Muchenje et al. 2015). So far, no other dietary efforts have been successful.

A more promising approach to decrease jejunal pH could be the application of probiotic strains such as lactic acid bacteria or *Bifidobacteria*. For instance, *Bifidobacterium breve* was shown to decrease faecal pH levels in humans (Ishikawa, Matsumoto et al. 2011). In laying hens, similar results were obtained by administering *Lactobacillus plantarum* metabolites (Choe, Loh et al. 2012). Such effects can be explained by the bacteria´s ability to produce organic acids such as lactate, acetate, propionate and butyrate (which can also be observed when diets contain high amounts of fibre). However, it should be kept in mind that probiotic strains colonize the large rather than the small intestine. Reliable studies proving pH changes in the small intestine due to probiotic strains are lacking. Hence, more research is needed to elucidate whether probiotics are able to reduce pH also in the jejunum and whether this could be sufficient to induce *gadX* expression in EPEC.

As described in Chapter 2.1.3, many drugs and compounds are available which interfere with the GABA metabolism in humans. Therefore, the development of compounds interacting with the bacterial GABA metabolism seems feasible. Such compounds could also be of bacterial origin. Bacterial metabolites are used as intra- and inter-species signalling molecules enabling the bacterium to sense the surrounding environmental conditions (such as cell population density) and to respond with appropriate strategies (as for example changes in virulence, biofilm

formation or motility) (Miller and Bassler 2001). Hence, in a well-balanced polyspecies community with accumulation of certain bacterial metabolites, even pathogens might become innocuous for the host.

So far, it has not been fully elucidated which metabolites are acting on which virulence regulators. One of the most extensively studied molecules in quorum sensing between different bacterial species is the autoinducer-2 (AI-2) which is widely distributed in various bacterial species. A well-established example of AI-2-mediated quorum sensing is the activation of luminescence in some marine *Vibrio* strains, which has been discovered more than 40 years ago and is still of interest because luciferase enzymes are an important tool in biological research. The AI-2 synthase is encoded on the gene *luxS* which exists in many Gram-negative bacteria such as *E. coli*, *Pseudomonas aeruginosa* or *Salmonella typhimurium* as well as in some Gram-positive bacteria as *Bacillus subtilis* (Bassler, Greenberg et al. 1997). Usually they do not only produce AI-2 but also express AI-2 transporter proteins that enable them to take up extracellular AI-2 secreted by surrounding bacteria. Even epithelial cells were shown to produce an AI-2 mimic, which seems surprising at first sight but is believed to be a response to bacterial stimulation (Ismail, Valastyan et al. 2016).

In *E. coli*, AI-2 was shown to positively control the biofilm formation by enhancing transcription of motility genes (Gonzalez Barrios, Zuo et al. 2006). Moreover, in pathogenic *E. coli* such as EPEC, EHEC and ETEC, AI-2 is strongly involved in virulence gene expression of LEE (Sircili, Walters et al. 2004, Bansal, Jesudhasan et al. 2008, Wang, Li et al. 2016). It is not clear how AI-2 influences virulence in *E. coli*, but there is evidence that this pathway is independent from PER (Sircili, Walters et al. 2004). It remains challenging to elucidate whether AI-2 could even modulate expression of genes outside LEE as for example *gadX*.
Several compounds are known to inhibit AI-2, thereby leading to a reduced virulence of EPEC and EHEC. Among those compounds the most effective ones are furanones, which were isolated from the algae *Delisae pulchra* and their sulphur derivates, thiophenones. Thiophenones are not only able to interfere with EPEC biofilm formation, they were also observed to decrease the expression of *eae* genes (Witso, Benneche et al. 2014, Witso, Valen Rukke et al. 2016). However, the knowledge of the underlying mechanisms is limited as it has not been studied whether such compounds have an impact on GABA production or whether they possibly directly interact with *gadX.* The mode of action of thiophenones is not clear yet but it is supposed that they act on genes which are required for the function of AI-2. From what

is known about similar systems in other species, there is reason to believe that the reduced *eae* expression upon AI-2 inhibition is embedded in a complex intracellular regulation process.

Until now, there is only limited research about particular compounds derived by the microbiota of healthy subjects which could interfere with virulence regulation of EPEC. De Sablet et al. revealed that molecules secreted by human digestive microbiota were able to inhibit EHEC virulence gene expression and Shiga toxin secretion (de Sablet, Chassard et al. 2009). Strikingly, this virulence repression was not related to known quorum sensing systems such as the *AI-2* pathway. Such molecules could be worth to be evaluated in more detail for their mode of action and their potential to interact with *gadX*.

Nitric oxide is supposed to have antimicrobial activity in many bacterial species (Fang 1997) and was shown to activate *gadX* expression in EHEC. This is believed to be carried out through an indirect inhibition of another transcription factor, the nitrite-sensitive repressor *NsrR,* which appears to be involved in LEE regulation and *gadX* expression and which is also present in other pathogenic *E. coli* strains (Rodionov, Dubchak et al. 2005, Bodenmiller and Spiro 2006, Branchu, Matrat et al. 2014). Intestinal epithelial cells are a main source of NO in the gut (Salzman 1995). Astonishingly, *L. rhamnosus* was found to be able to induce NO synthesis in human intestinal cell cultures (Korhonen, Korpela et al. 2001). It is tempting to speculate that the stimulation of NO synthesis in intestinal cells by *L. rhamnosus* inhibits NsrR expression, thereby leading to an enhanced *gadX* expression in EPEC. As a result, EPEC virulence would decline while GABA production would be elevated, leading to improved mucosal barrier functions of the gut (e.g. increased MUC1 expression). This hypothesis is supported by the above-mentioned study of Mao et al., who found an enhanced MUC1 expression of the intestinal epithelium of weaned piglets supplemented with *L. rhamnosus*. However, this would also be in line with the above-mentioned assumption that NO can induce mucin expression. Nevertheless, the fact that *L. rhamnosus* is supposed to have antimicrobial activity against EPEC (Davoodabadi, Soltan Dallal et al. 2015) contributes to the hypothesis that there might indeed be a direct link between EPEC virulence and *L. rhamnosus*.

Even though there are various plausible theories for the effects of probiotics on gut health, it seems worth considering that GABA could be a crucial player in the complex feedback system between bacteria and host. Additionally, the fact that there are probably many bacteria-derived molecules involved in quorum sensing which have not been identified yet, provides a chance to discover compounds that could act on *gadX* expression.

Many authors see the modulation of bacterial behaviour such as that of EPEC within polyspecies communities as the most viable future approach to combat enteric diseases. There might be a new dimension ahead that could dismiss the idea of the formerly common practice to divide bacteria in good and bad. In fact, the ability to modulate the behaviour of pathogens could lead to a breakthrough in the development of new pharmaceuticals. However, more research is necessary to understand the complex communication system between bacteria and host.

Conclusions

According to literature, there is strong evidence suggesting that moderate levels of GABA have a positive impact on the gut – affecting motility as well as immunological factors. However, the direct effect of EPEC-derived GABA on the epithelium might mainly be relevant for the jejunum, where GABA levels are usually low. In the own experiments described in Chapter 4, MUC1 expression was increased in jejunal epithelium *ex vivo* after topical GABA application, suggesting that GABA may positively affect mucosal protective mechanisms besides its well-described effects on gastrointestinal motility. In a second study, presented in Chapter 5, *gadX* stimulation lead to significant reduction of virulence and increased production of GABA in EPEC. Hence, by stimulating bacterial GadX expression, the host would not only benefit from a weakened bacterial pathogenicity but also from increased jejunal GABA levels which could lower the intestinal susceptibility to different environmental and infectious stressors.

Merging the results of both studies offers the tempting conclusion that GadX could indeed become a relevant target for the treatment and prophylaxis of diarrhoeic diseases, at least when EPEC are involved in their etiology. Either the indirect stimulation of GadX by acidifying the jejunal pH through probiotics or the direct stimulation of the *gadX* gene by chemical compounds or bacterial metabolites could be a promising approach that could contribute to fighting diarrhoeic diseases.

Finally, the results of this work support the growing body of evidence that the similarity between bacterial metabolites and mammalian neurotransmitters is not a coincidence but an important factor of the astonishingly complex communication system between host and gut microbiota. In view of the facts that the most abundant enteric bacteria are able to produce the most relevant mammalian neurotransmitter in sufficient amounts and that GABA receptors are universally expressed by the intestinal epithelium, the hypothesis of a bidirectional communication system between host and gut microbiota with GABA as a key player appears

strikingly convincing. Although it will remain challenging to elucidate the multifaceted regulation processes, future research in this field may provide promising opportunities for new prevention strategies against diarrhoea.

7. Summary

Summary of the PhD Thesis "GABA, GadX and Gut Health

How Stimulation of EPEC Transcription Factor GadX Can Improve the Gut Mucosal Barrier"

Diarrhoeic diseases belong to the most common health issues world-wide. They are a threat to both mankind and animals and are often induced by bacteria such as enteropathogenic *Escherichia coli* (EPEC). During the past decades, antibiotics have been the treatment of choice. The recent restriction of in-feed antibiotics raises problems particularly in pig fattening where postweaning diarrhoea causes significant losses. Thus, there is an increasing demand for alternative approaches to prevent postweaning diarrhoea.

Growing evidence suggests that the inhibitory transmitter γ-aminobutyric acid (GABA) has not only functions in the central nervous system but is also an important player in the gut where it affects motility and immune functions. Besides that, the virulence gene regulation of EPEC is closely linked to GABA production, a process involving the bacterial transcription factor GadX. Moreover, GABA is contained in a regular diet as it is a significant component of the free amino acid pool of all kinds of plants, is available in dairy products and can be produced by several different bacteria during food or feed fermentation.

The major goals of this thesis were to:

a) prove that an activation of the transcription factor GadX in EPEC would increase GABA production
b) test whether an overexpression of the transcription factor *gadX* in EPEC would lead to reduced virulence factor gene expression and reduced virulence *in vitro*
c) examine whether topical GABA application would have a positive effect on the mucosal barrier of the intestinal epithelium of pigs *ex vivo*, thereby potentially decreasing the susceptibility to gut infections
d) place the results in a broader, translational context with respect to their relevance for future approaches to fight diarrhoea.

Two studies were performed addressing these questions. Methods and results were presented in the manuscripts „GABA selectively increases mucin-1 expression in isolated pig jejunum" (Genes & Nutrition, 2015, 10(6):47) and "The GadX regulon affects virulence gene expression

and adhesion of porcine enteropathogenic Escherichia coli *in vitro*“ (Veterinary and Animal Science, 2017, 3:10-17).

In the first study, the influence of GABA on porcine intestinal epithelium was evaluated in an *ex vivo* approach. Isolated porcine jejunal mucosa was incubated with different concentrations of GABA or its precursor glutamine on the luminal side for 4 hrs. Changes in the mRNA expression levels of mucins, $GABA_B$ receptor, enzymes involved in the metabolism of GABA and glutamine, interleukin-10 and glutathione peroxidase 2 were analysed by RT-qPCR. It was observed that the expression of mucin-1 on mRNA and protein level was selectively upregulated by the treatment with GABA, indicating that GABA can directly enhance mucosal barrier functions without vagal involvement.

In the second study, the role of *gadX* was studied with respect to crucial virulence gene expression in EPEC, the latter being isolated from diarrhoeic pigs. The transcription factor *gadX* was either stimulated through acidification or by overexpression via an inducible plasmid. In both approaches it was shown that GABA production was significantly increased upon *gadX* induction while mRNA expression of the virulence gene intimin was reduced. Furthermore, an adhesion test demonstrated that strains transformed with the *gadX* plasmid completely lost their ability to attach and adhere to an intestinal porcine epithelial cell line.

Finally, the potential of GABA and *gadX* as target for the prevention of diarrhoea is discussed and several approaches to stimulate *gadX* expression *in vivo* are introduced. It is concluded that *gadX* stimulation in EPEC might not only decrease virulence gene expression but could also exert an infection-preventing effect by increasing bacterial GABA production and thereby enhancing intestinal mucin expression. Beyond that, the results of this work support the hypothesis that GABA is part of the strikingly complex communication system between host and gut microbiota and could be vital to maintain the sensitive balance of the interactions between microbiota and host. However, more research is required to evaluate the underlying pathways and to identify potential targets which could be used to activate GABA production and *gadX* expression *in vivo.*

8. Zusammenfassung

Zusammenfassung der PhD-Arbeit "GABA, GadX und Darmgesundheit:

Wie eine Stimulation des EPEC Transkriptionsfaktors GadX die Barriere der Darmschleimhaut verbessern kann"

Durchfallerkrankungen gehören zu den häufigsten Gesundheitsproblemen weltweit. Sie stellen eine Bedrohung für Mensch und Tier dar und werden häufig durch Bakterien wie z. B. enteropathogene *Escherichia coli* (EPEC) verursacht. Während der letzten Jahrzehnte war die Therapie mit Antibiotika Mittel der Wahl. Da jedoch bei Bakterien zunehmend Resistenzen gegen Antibiotika auftraten, begannen viele Länder den Einsatz von Antibiotika stark zu reglementieren. Die daraus resultierenden Restriktionen im Bereich der Fütterungsantibiotika verursachen insbesondere in der Schweinemast zunehmend Probleme, da die Absatzferkel-Diarrhö zu massiven Ferkelverlusten führt. Aus diesem Grund steigt die Nachfrage nach alternativen Lösungen zur Prävention der Absatzferkel-Diarrhö.

Es gibt zunehmend Hinweise darauf, dass der inhibitorische Transmitter γ-Aminobuttersäure (GABA) nicht nur im Zentralnervensystem eine Rolle spielt, sondern auch als wichtiger Botenstoff im Darm fungiert, wo er Motilität und Abwehrfunktionen beeinflusst. Die Regulation der Virulenz von EPEC, welche den der bakteriellen Transkriptionsfaktor GadX involviert, ist eng mit der Produktion von GABA verbunden. Darüber hinaus gehört GABA zur regulären Ernährung, da es eine Hauptkomponente des freien Aminosäurepools von Pflanzen darstellt, in Milchprodukten enthalten ist und von verschiedenen, fermentativ genutzten Bakterienarten synthetisiert werden kann.

Die Hauptziele der vorliegenden Arbeit waren:

a) zu überprüfen ob die Aktivierung des Transkriptionsfaktors GadX zu einer verstärkten GABA-Produktion in EPEC führt
b) zu testen ob die Aktivierung des Transkriptionsfaktors GadX in EPEC eine verminderte Virulenz *in vitro* bewirkt
c) zu untersuchen, ob eine lokale Applikation von GABA *in vitro* einen positiven Effekt auf die Schleimhautbarriere im Darmepithel von Schweinen hat und dadurch möglicherweise die Anfälligkeit für Darminfektionen verringert
d) die Ergebnisse im Hinblick auf die Relevanz für zukünftige Strategien zur Bekämpfung von Durchfallgeschehen bei verschiedenen Spezies in einen Zusammenhang zu setzen

Um diesen Fragenstellungen nachzugehen, wurden zwei Studien durchgeführt. Die Methoden und Ergebnisse sind in den Manuskripten „GABA selectively increases mucin-1 expression in isolated pig jejunum“ (Genes & Nutrition, 2015, 10(6):47) und “The GadX regulon affects virulence gene expression and adhesion of porcine enteropathogenic Escherichia coli *in vitro*“ (Veterinary and Animal Science, 2017, 3:10-17) zusammengefasst.

In der ersten Studie wurde in einem *ex vivo* Ansatz der Einfluss von GABA auf das Epithel vom Schweinedarm untersucht. Dafür wurde isolierte Jejunum-Schleimhaut vom Schwein für vier Stunden mit verschiedenen Konzentrationen von GABA bzw. dessen Vorstufe Glutamin inkubiert. Anschließend wurde mittels RT-qPCR die mRNA-Expression von Muzinen, dem $GABA_B$ Rezeptor, der in den GABA- und Glutamin-Stoffwechsel involvierten Enzyme, von Interleukin-10 und der Glutathionperoxidase 2 untersucht. Es zeigte sich, dass durch die Behandlung mit GABA die Expression von Muzin-1 sowohl auf mRNA- als auch auf Proteinebene signifikant hochreguliert wurde. Daraus kann geschlussfolgert werden, dass GABA auch ohne Beteiligung des *Nervus vagus* direkt die Funktionen der Schleimhautbarriere verbessern kann.

Im zweiten Artikel wurde die Auswirkung von *gadX* auf die Expression entscheidender Virulenzgene von Schweine-adaptierten EPEC-Stämmen untersucht. Der Transkriptionsfaktor *gadX* wurde entweder durch Ansäuerung des Milieus oder durch Überexpression eines induzierbaren GadX-tragenden Plasmids stimuliert. In beiden Ansätzen stieg die GABA-Produktion signifikant an, während gleichzeitig die mRNA-Expression des Virulenzgens Intimin herunterreguliert wurde. Des Weiteren konnte in einem Adhäsionstest gezeigt werden, dass Stämme, die mit dem GadX-Plasmid transformiert worden waren, ihre Fähigkeit zur Anlagerung und Adhäsion an eine porzine Darm-Zelllinie vollständig verloren.

Das Potential von GABA und *gadX* für die Prävention von Durchfallerkrankungen wird diskutiert und verschiedene Ansätze vorgestellt, wie die *gadX*-Expression *in vivo* stimuliert werden könnte. Es wird geschlussfolgert, dass die Stimulation von *gadX* in EPEC nicht nur die Expression von Virulenzgenen reduziert, sondern auch dazu beitragen kann, eine Infektion zu verhindern, indem die bakterielle GABA-Synthese verstärkt und gleichzeitig die Muzin-Expression der Darmschleimhaut verbessert wird. Darüber hinaus unterstützen die Ergebnisse dieser Arbeit die Hypothese, dass GABA Teil des bemerkenswert komplexen Kommunikationssystems zwischen Wirt und Darmflora sein könnte und eine essentielle Bedeutung für die Aufrechterhaltung des empfindlichen Gleichgewichts beim Zusammenspiel

zwischen Mikrobiota und Wirt hat. Allerdings ist noch mehr Forschung in diesem Bereich nötig, um potentielle Targets zu identifizieren, die die GABA-Produktion sowie die *gadX*-Expression *in vivo* aktivieren könnten.

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List of Publications

Publications (peer reviewed)

2017

Braun, H. S.; Sponder, G.; Aschenbach, J. R.; Kerner, K.; Bauerfeind, R.; Deiner, C.
The GadX regulon affects virulence gene expression and adhesion of porcine enteropathogenic Escherichia coli *in vitro*.
Veterinary and animal science; 3, p. 10–17

2015

Braun, H.-S.; Sponder, G.; Pieper, R.; Aschenbach, J. R.; Deiner, C.
GABA selectively increases mucin-1 expression in isolated pig jejunum.
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Abstracts in proceedings & participations in conferences

2015

Braun, H.-S.; Deiner, C., Pieper, R.; Aschenbach, J. R.
Physiological GABA levels in the caecum of pigs are sufficient to ensure proper mucin expression.
13th Digestive Physiology in Pigs Symposium - Kliczków – 19.05.-21.05.2015.
In: 13th Digestive Physiology in Pigs Symposium Kliczków

2014

Braun, H. S.; Sponder, G.; Aschenbach, J. R.; Kerner, K.; Bauerfeind, R.; Deiner, C.
The influence of GadX on virulence factor expression and GABA production of porcine EPEC: an RT-qPCR approach.
21. DVG Tagung der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft - Zürich – 13.02.-15.02.2014.
In: 21. Tagung der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft – Deutsche Veterinärmedizinische Gesellschaft (Hrsg.)
München: Ludwig-Maximilians-Univ. S. V33

Deiner, C.; Braun, H. S.; Sponder, G.; Pieper, R.; Zentek, J.; Aschenbach, J. R.
GABA selectively increases mucin-1 expression in isolated pig jejunum.
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2012

Braun, H.S.; Pieper, R.; Tietjen, U.; Wolf, K.; Aschenbach, J. R.; Deiner, C.

Luminal surplus of tryptophan stimulates the kynurenine pathway in the jejunal mucosa of pigs ex vivo.
7. Doktorandensymposium und DRS-Präsentationsseminar “Biomedical Sciences“ am Fachbereich Veterinärmedizin der Freien Universität Berlin 13.07.2012.

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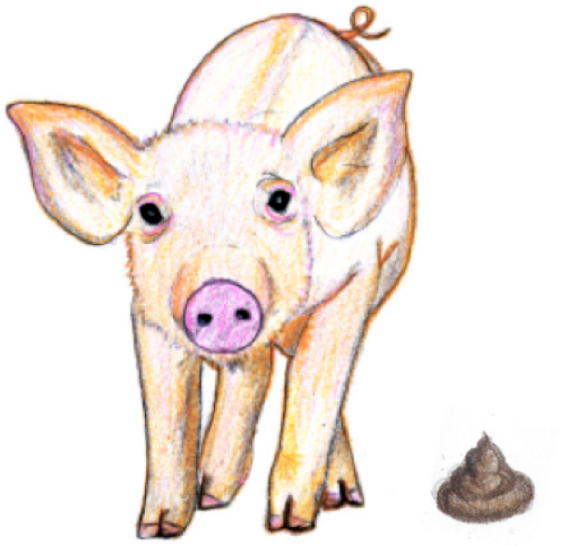
Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 15.01.2019 **Hannah-Sophie Braun**



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