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The Intestinal Mucosal Network in the Pig:

A Histological View on Nutrition-Microbiota-Pathogen-Host-Interactions

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“Time is a drug. Too much of it kills you.”

— Terry Pratchett, Small Gods

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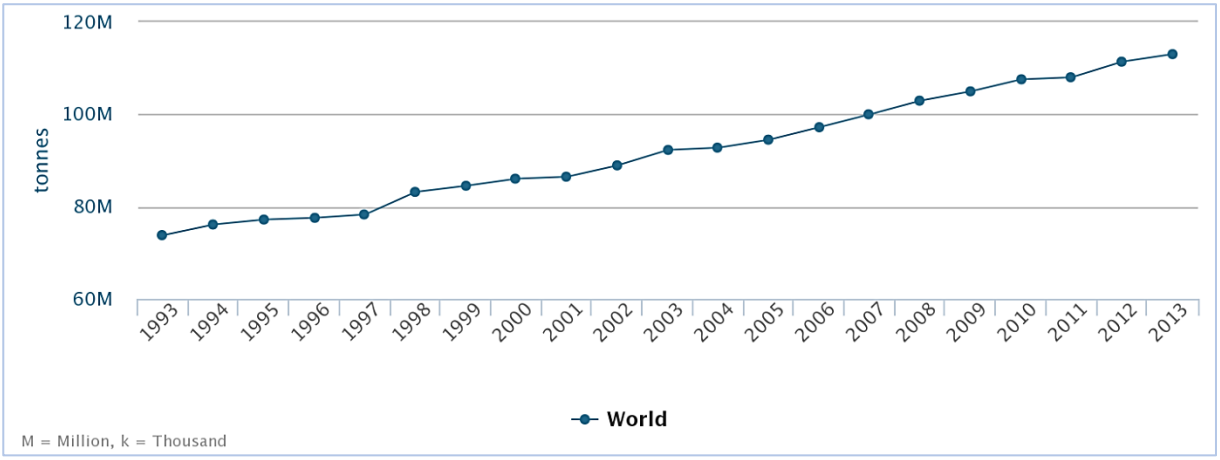
List of abbreviations

ANOVA	Analysis Of Variance
Aq. Dest / Aq. Bidest	Distilled / Double-Distilled Water
BSA	Bovine Serum Albumin
CFU	Colony-Forming Unit
DAB	3,3'-Diaminobenzidine
DPI	Day Post Infection
dpw	Days post weaning
E. faecium	*Enterococcus faecium* NCIMB 10415
FACS	Fluorescence-Activated Cell Sorting (a specialized type of flow cytometry)
FAE	Follicle Associated Epithelium
GALT	Gut Associated Lymphoid Tissue
H&E	Haematoxylin and Eosin Stain
HRP	Horseradish Peroxidase
IEL	Intraepithelial Lymphocyte
IHC	Immunohistochemistry
IL	Interleukin
LP	Lamina Propria
LPS	Lipopolysaccharides
MALT	Mucosa Associated Lymphoid Tissue
MCs	Mast Cells
mRNA	messenger Ribonucleic Acid
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
real-time PCR	real-time Polymerase Chain Reaction
RT	Room Temperature
S.Typhimurium	*Salmonella enterica* serovar Typhimurium
SD	Standard Deviation
SG	Significance Groups
SIV	Swine Influenza Virus
TBS	Tris Buffered Saline
TGEV	Transmissible Gastroenteritis Coronavirus
TGF-β	Transforming Growth Factor Beta
TLR	Toll-Like Receptor

Tregs	T regulatory cells
ZSF	Zinc Salt Fixation

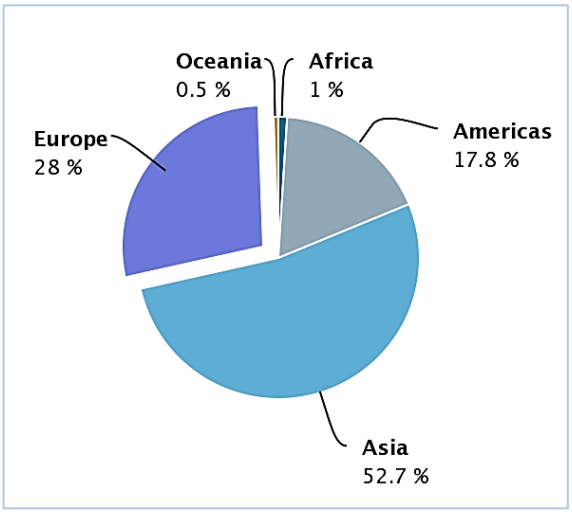
1. General introduction

The pig is one of the most important farm animals and pork production has a huge economic impact worldwide. According to FAOSTAT (Food and Agriculture Organization of the United Nations - Statistics Division; http://faostat3.fao.org/browse/Q/QL/E), there has been a steady rise in worldwide pork production, which reached about 113 million tons in 2013. In this production, Europe's share is about 28% and Germany ranges among the top 5 producers of pig meat worldwide (Figure1-1).



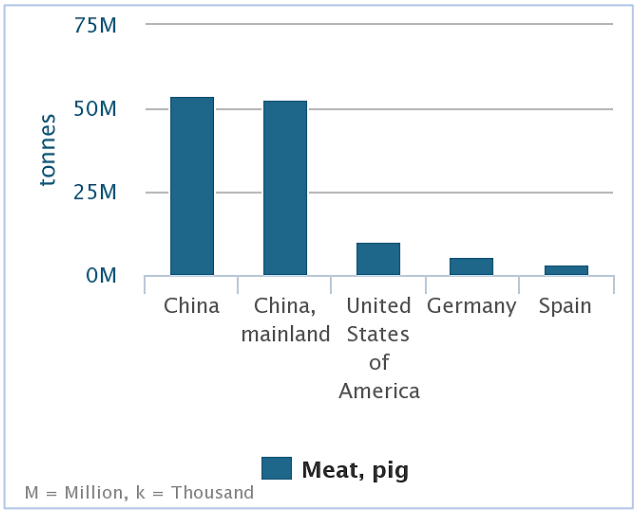
Pig meat production worldwide 1993 - 2013

[© FAO] [2015] [FAOSTAT Domains; Livestock Primary; Meat, pig, Production share by region 1993 - 2013] [http://faostat3.fao.org/browse/Q/QL/E] [2015-04-05]



Pig meat production worldwide
Production share by region
Average 1993 - 2013

[© FAO] [2015] [FAOSTAT Domains; Livestock Primary; Meat, pig, Production share by region1993 - 2013] [http://faostat3.fao.org/browse/Q/QL/E] [2015-04-05]



Production of top 5 producers of pig meat worldwide 2013

[© FAO] [2015] [FAOSTAT Domains; Livestock Primary; Meat, pig, Production of top 5 producers 2013] [http://faostat3.fao.org/browse/Q/QL/E] [2015-04-05]

Figure 1-1: Pig meat production worldwide

Animal husbandry under the viewpoint of economic efficiency on such a large scale is commonly carried out under industrial conditions. This means that modern swine production is a highly engineered system and production levels increased substantially in the last 40 years while the need for manual labor was reduced (Kittawornrat & Zimmerman, 2011). Intensive pig husbandry involves gathering high numbers of animals in relatively small areas, resulting in a high output of animal product per unit surface area. An effect on the local environment is a high nitrogen and phosphorus accumulation (Menzi *et al*, 2010). Animals in intensive livestock production systems are kept in high stocking density and have to put up with several unfavourable factors, amongst them crowding stress, hierarchy fights and infection pressure (de Groot *et al*, 2001; Menzi *et al*, 2010; Khafipour *et al*, 2014). For a long time, antibiotic growth promotors (in-feed-antibiotics) were used to compensate for some of these negative effects. It is now generally accepted that the overly intensive and uncontrolled use of antibiotics affects human as well as animal health due to a rise in multiple resistances of bacterial infections. Additionally, consumers have become increasingly concerned about drug residue in meat products (Thacker, 2013). Consequently, the use of in-feed-antibiotics has lately been viewed critically and their usage as feed additives was banned completely in the EU in 2006 (Regulation (EC) No. 1831/2003).

In the face of this, it seems plausible that improvements in pig nutritional strategies are of great value and an intensive amount of research is focused on the development of alternatives to antibiotics to maintain swine health and performance. According to Thacker (2013) such alternatives include probiotics, prebiotics, enzymes, acidifiers, plant extracts and nutraceuticals such as copper and zinc as well as some less traditional alternatives. Unfortunately, most of these compounds yield inconsistent results and rarely equal antibiotics in their effectiveness. Therefore, research is still needed in this area and the perfect alternative does not seem to exist until now (Thacker, 2013).

The pig is not only an important farm animal, it is also of great importance in biomedical, pharmaceutical and medicine research. Its organism has several biological similarities to the human one and therefore, it is an important experimental animal, a model system for biological research and a subject for medical and pharmaceutical investigation and training (de Almeida & Bendixen, 2012). Also, in human nutrition and healthcare, alternatives for antibiotics or anti-inflammatory drugs are attracting ever more interest, for example the use of probiotics, which have recently been a much-discussed topic. However, the mode of action of probiotics is poorly understood. Additionally, reported mechanisms of probiotic actions are often the results of *in vitro* experiments and therefore, these results should be confirmed by *in vivo* studies (Oelschlaeger, 2010).

This thesis was designed as a part of the collaborative research centre (German: Sonderforschungsbereich) “SFB 852”, where different feeding strategies for pigs were

examined *in vitro* and *in vivo*. The scientific aim of the SFB 852 “Nutrition and intestinal microbiota - host interactions in the pig” was to better understand how nutritional factors influence the functioning of the intestine in the pig. This question was addressed by a total of 15 sub-projects using a multitude of analytical, molecular biological and bioinformatic methods. Research efforts were focused on feed additives and, in particular, on mechanistic studies on the effects of probiotics as well as the trace mineral zinc. The subproject C2 “Core facilities: Central technique and bioinformatic toolbox” provided the members of the SFB with techniques specifically adapted to the pig.

The field of work for this thesis was the “Histology Platform” within subproject C2 with the purpose to apply and refine a broad spectrum of histological methodologies to the porcine organism in the course of several feeding trials with pigs. The effects of different nutritional strategies on histological parameters under normal housing conditions or in challenging situations were examined. The intestine as interface between host, nutrition and microbiota as well as their intense interaction was the focus of research.

A particularly important aspect of the research work was the joining of histological knowledge with additional methodological approaches in team with researchers of the SFB 852. By joining branches of the field of biomedical sciences, a picture of the processes in intestinal networks was to be gathered in more detail. Therefore, several smaller projects were completed within this thesis, contributing to other publications.

2. Literature review

2.1. The pig as a model organism in biomedical sciences

In the field of biomedicine, the pig represents a remarkable and promising model organism helping to decipher questions of basic as well as applied research (Aigner *et al*, 2010; Stramandinoli-Zanicotti *et al*, 2014). Because pigs are anatomically, physiologically and genetically comparable to humans, they are suitable for studies of human nutrition and health problems (Guilloteau *et al*, 2010; Verma *et al*, 2011; Whyte & Prather, 2011; Prather *et al*, 2013; Wolf *et al*, 2013). Especially the resemblance on the intestinal level between humans and pigs is advantageous (Gonzalez *et al*, 2015). Although mice are the prominent model in intestinal research allowing extensive studies on e.g. the gut microbiota, translation of results to human beings has to be done carefully, so the pig is a convincing alternative (Nguyen *et al*, 2015). Pigs also provide an experimental animal model to study developmental immuno-toxicology (Rothkötter *et al*, 2002), innate immunity and disease (Fairbairn *et al*, 2011) and population-scale disease dynamics (Lanzas *et al*, 2010). Moreover, the pig is the most relevant animal model for studying the intestinal effects of food and feed contaminants like the mycotoxin deoxynivalenol (Kolf-Clauw *et al*, 2009). Nevertheless, the pig model has its shortcomings. An example for that would be a comparative study on the anatomy of humans and pigs showing that significant differences exist between human and porcine intestines e.g. in vascular anatomy (Trotha *et al*, 2015). This should be kept in mind when interpreting and comparing results.

2.2. Porcine intestinal morphology, physiology and immunology

2.2.1. Macroscopic and microscopic anatomy of the porcine intestinal tract

The pig is an omnivorous, monogastric mammal with a compartmentalised stomach. Macroscopically, the gut follows the general structure found in all domestic mammals (Figure 2.2.1-1). It is divided into the small intestine, consisting of *duodenum*, *jejunum* and *ileum* and the large intestine, consisting of *caecum, colon* and *rectum*. In the adult swine, the small intestine is ca. 18.5m and the large intestine ca. 5m in length. Typical for the pig is the arrangement of the *colon ascendens.* It is rolled up on itself, forming a conical mass of spiralling coils (Nickel *et al*, 2004).

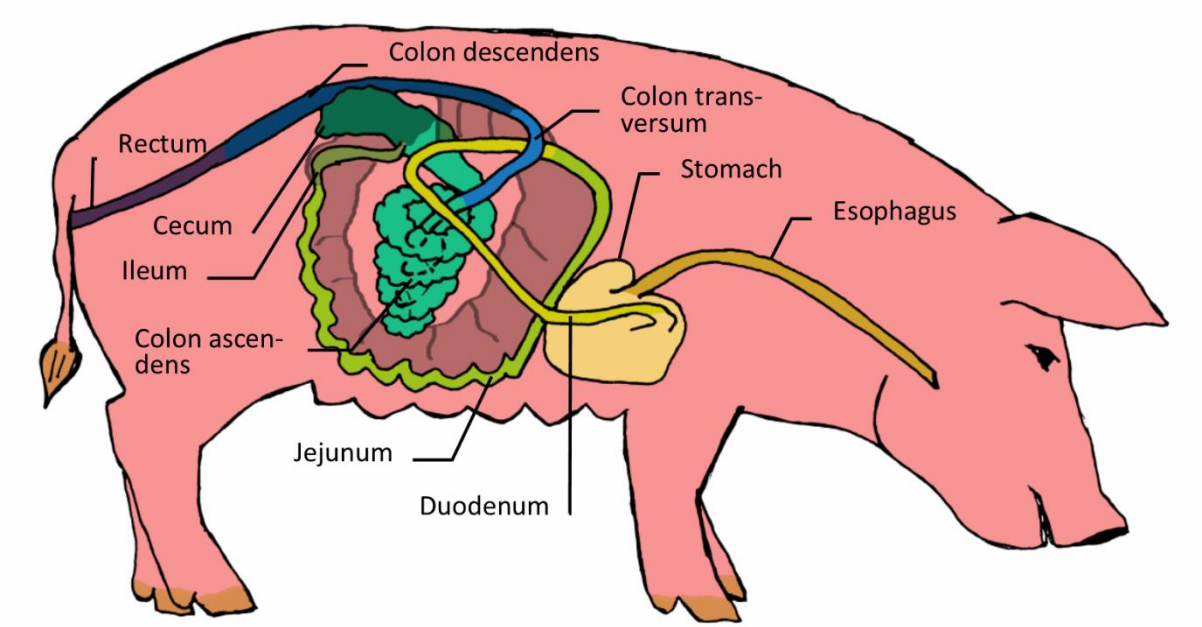


Figure 2.2.1-1: Macroscopic anatomy – Diagram of the gastrointestinal tract of the pig

Also on a microscopic level, the porcine gut wall follows the general structure found in the *Tubus alimentarius* of all domestic mammals. It consists of the following layers (from inside to outside): *Tunica mucosa* - consisting of *Epithelium mucosae*, *Lamina propria mucosae* and *Lamina muscularis mucosae*; *Tela submucosa; Tunica muscularis* - consisting of *Stratum circulare* and *Stratum longitudinale; Tela subserosa and Tunica serosa* - consisting of *Lamina propria serosae* and *Epithelium serosae* (Weyrauch *et al*, 2009).

There are several structural features of the mucosa and submucosa enlarging the absorptive surface of the small intestine. The *plicae circulares* are a series of circular folds, lined by finger-like protrusions - the villi. The villi are covered by the enterocytes, which in turn have epithelial membrane extensions - the microvilli. The epithelium of each villus continues into tubular glands, the crypts of Lieberkühn (Mescher, 2009). The single-layered epithelium in the intestinal tract mainly consists of four cell types, which are all descendants of stem cells residing in the base of the crypts, allowing a continuous cell turnover: > 80% columnar shaped absorptive cells, ~4 (duodenum) - 16% (colon) mucus producing goblet cells, <1% antimicrobial peptides producing Paneth cells (existence of this cell population is under discussion for the pig (Myer, 1982; van Es & Clevers, 2014)) and ~ 1% hormone producing enteroendocrine cells (van der Flier & Clevers, 2009). The lamina propria forms the supporting layer of the epithelium, consisting of connective tissue and supplying vessels, and is populated by extracellular matrix producing cells, neurons, smooth muscle fibres and a vast amount of immune cells (Liebich, 2010). The muscularis mucosa ensures local movement of villi and

circular folds. In the submucosa, vessels and nerve plexuses form a supplying network for the overlying structures. In the submucosa of the duodenum, glands (Brunner's glands) can be found, secreting alkaline mucus into the lumen, and in the jejunum and ileum, lymphoid nodule aggregates (Peyer´s patches) are to be found, which extend from the submucosa into the lamina propria (Mescher, 2009; Liebich, 2010). The muscular layer is important for peristalsis, contains nerve plexuses and is covered by the serosa, which also forms the supporting mesentery and holds connective tissue, large vessels, nerves and lymph nodes.

Porcine lymph nodes display a special structure, which is termed “inverted”. Interestingly, they share this feature with hippopotamuses, rhinoceroses, elephants and some marine mammals (Rothkötter, 2009; Tizard, 2013). Rothkötter (2009) and Tizard (2013) describe the structural arrangement thus: The lymph nodes consist of several lymphoid nodules, which are surrounded by the commonly internal “medullary tissue”. Each lymphoid nodule is supplied by a single afferent lymphatic vessel. This lymphatic vessel is not ending in a superficial cortical sinus but penetrates deep into the lymph nodules centre. From this lymphatic vessel, the arriving tissue fluid and cells drain into a surrounding sinus. From there, the structure is similar to the lymph node in other species. Adjacent to the sinuses are B cell areas with follicles (cortex) and surrounding T cell areas (paracortex) with high endothelial venules. The medullary tissue surrounds these lymphatic-sinus-cortex-paracortex aggregations and is often shared by adjacent nodules. Lymph passes from the cortex at the centre of the nodule to the medulla at the periphery before leaving through the efferent vessel that drains the medulla. While immigration of cells into the lymph node is similar to that seen in other mammals, where they either enter by afferent lymph vessels or by high endothelial venules, lymphocytes exit the porcine lymph node mainly via high endothelial venules and not via efferent lymph vessels. As a result, low numbers of lymphoid cells migrate through the medulla and there are only low numbers of lymphoid cells in the thoracic duct of pigs (Rothkötter, 2009; Tizard, 2013).

The micro-anatomical structure of the large intestine in general follows the organisation of the one described for the small intestine. An important difference is the absence of villi and the higher amount of goblet cells (Lewis & Lee Southern, 2000; van der Flier & Clevers, 2009).

It is important to note that normal age-dependant development, artificial weaning and different feeding regimes are associated with morphological changes in the porcine intestine (Al Masri *et al*, 2015).

2.2.2. Physiology of the porcine intestinal tract

Like the anatomy, the physiology of the intestinal tract of the pig is generally comparable to other domestic mammals. Mosenthin (1998) specifically described aspects of porcine

nutritional physiology: The main function of the intestinal tract is the digestion and absorption of nutrients. The small intestine is the principal organ for this purpose, although digestion also occurs in the hind gut through fermentation. The goal is to first convert the nutrients into an absorbable form and secondly transport them from the gut lumen across the epithelial membrane into the blood or lymphatic system. The epithelial surface amplification already described above increases the contact area between nutrients and absorptive cells and thereby multiplies digestive efficiency. A key element of this contact area is the brush border membrane. It consists of microvilli and a glycoprotein coating - the glycocalyx. The brush border membrane contains enzymes, crucial for the carbohydrate and protein digestion, and carrier proteins for the absorption of nutrients (Mosenthin, 1998).

Digestion is initiated as soon as feed enters the oral cavity. A large arsenal of secreted substances from salivary glands, stomach and the intestinal glands as well as liver and pancreas has already facilitated the decomposition of the feed before it reaches the brush border membrane. Thus, there is a phase of luminal digestion where the nutrients (carbohydrates and proteins) are broken down into smaller parts and a phase of mucosal digestion in the brush border, where these products are further broken down to be absorbed into the enterocytes and processed to be passed on into the blood. Dietary fats, after first being emulsified, diffuse into the enterocytes and are either rebuilt to chylomicrons to be transported via lymph or, in case of short-chain fatty acids, pass directly into the blood (Lewis & Lee Southern, 2000).

The large intestine reabsorbs water, electrolytes and other substances coming from the upper part of the intestine, excretes metabolic residue and provides a fermentation chamber for the resident microbiota, which enables the host to utilize otherwise non-digestible components (Mosenthin, 1998).

2.2.3. Immunology of the porcine intestinal tract

Since the intestinal tract is also an important interface between potentially pathogenic influences from outside or inside, another important aspect of intestinal biology is the resident immune system. To cope with antigens, evolution has created a highly effective and multifaceted defence system. As its first part, there are physical barriers like the intestinal mucus, the commensal flora and the epithelium. Second-level defence is the innate immune system, which rapidly reacts by cellular or acellular mechanisms such as inflammation; it has no memory function and uses preformed receptors to detect pathogens. Cells with important innate immune functions are macrophages, dendritic cells, granulocytes, natural killer cells and mast cells. Third-level defence is the adaptive immune system. It reacts rather slowly (within several days) to an infection, but recognizes and destroys invaders as well as learns in the process. The adaptive immune system generates great numbers of completely new and

unique receptors to detect pathogens. Cells with important adaptive immune functions are T and B cells. Many of the elements of the immune system mentioned so far have functions in more than one area, and there are various interconnecting pathways (Tizard, 2013).

As harmful and beneficial antigens meet in the intestine, the resident immune system has to discriminate between them. Although a strong protective immune response is necessary to protect the mucosal barriers from pathogen invasion, a balance must be kept in terms of responses against commensal bacteria or food antigens. Therefore, a complex regulatory network maintains homeostasis in this microenvironment (Mowat, 2003).

2.3. The intestinal mucosal network

Table 2.3-1 gives an overview on prominent cellular and acellular components of the mucosal network. Several of them will be described in the following chapters and set in relation to each other.

Figure 2.3-1 will be referred to in the following sections, illustrating some of the described processes.

Table 2.3-1: Prominent components (cellular and acellular) of the intestinal mucosal network

Gut lumen	▶ Ingesta / Nutrients ▶ Microbiota / Pathogens / Microbial metabolites ▶ Leukocytes ▶ Secretory IgA ▶ Antimicrobial peptides ▶ Mucus
Epithelium	▶ Glycocalyx ▶ Enzymes ▶ Absorptive enterocytes ▶ Secretory enterocytes ▶ Goblet cells ▶ Enteroendocrine cells ▶ (Paneth cells) ▶ Epithelial progenitor cells ▶ Leukocytes ▶ Intraepithelial lymphocytes ▶ Other leukocytes (depending on recruitment to the epithelium or into the gut lumen they may just traverse the epithelium) ▶ Basement membrane (produced from epithelium and underlying connective tissue cells)
Lamina propria	▶ Connective tissue cells (Fibrocytes / Fibroblasts) ▶ Extracellular matrix ▶ Intercellular fluid ▶ Blood vessels (composed of endothelial cells which are accompanied to differing degrees by smooth muscle cells, pericytes and nerves) ▶ Arterioles ▶ Capillaries (main place for exchange of CO_2/O_2, nutrients and water) ▶ Postcapillary venules (main location for exit of leukocytes and plasma protein) ▶ Venules ▶ Lymphatic system ▶ Prelymphatic channels ▶ Lacteals (composed of lymphatic endothelial cells) ▶ Smooth muscle cells of villus (important for villous pump mechanism) ▶ Aganglionic plexus of mucosa (for goblet cell secretion and villous pump) ▶ Leukocytes ▶ Dendritic cells ▶ Mast cells ▶ Macrophages ▶ Neutrophilic granulocytes ▶ Eosinophilic granulocytes (prominent cell population in the pig) ▶ B cells / Plasma cells ▶ T cells
Lamina muscularis mucosae	▶ Smooth muscle cells with aganglionic plexus of lamina muscularis mucosae and traversing vessels
“Everywhere”	▶ pH ▶ Concentration gradients ▶ Messenger molecules (e.g. cytokines) ▶ Transporter ▶ Cell junctions ▶ Ligands / Receptors ▶ Metabolic products

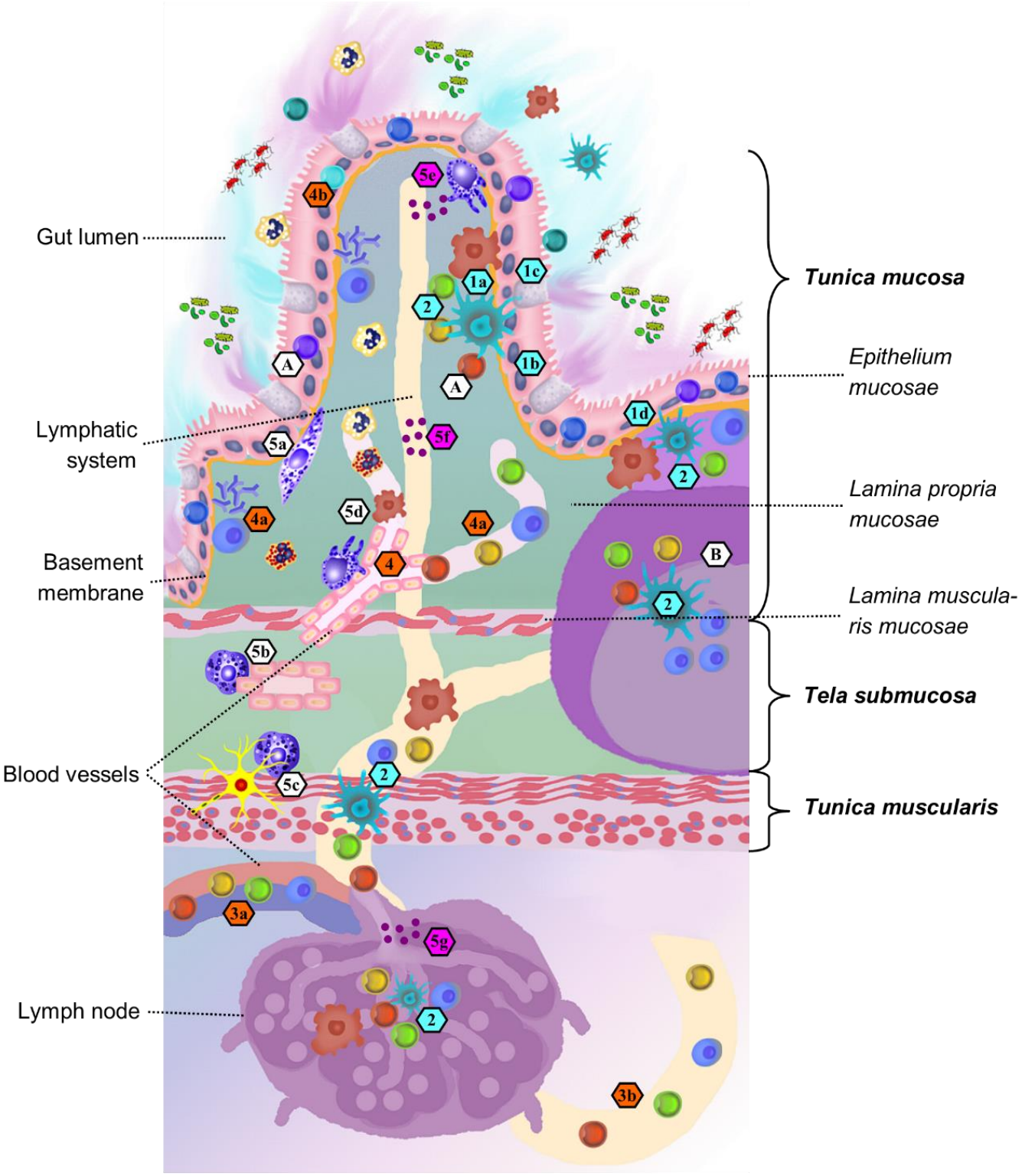
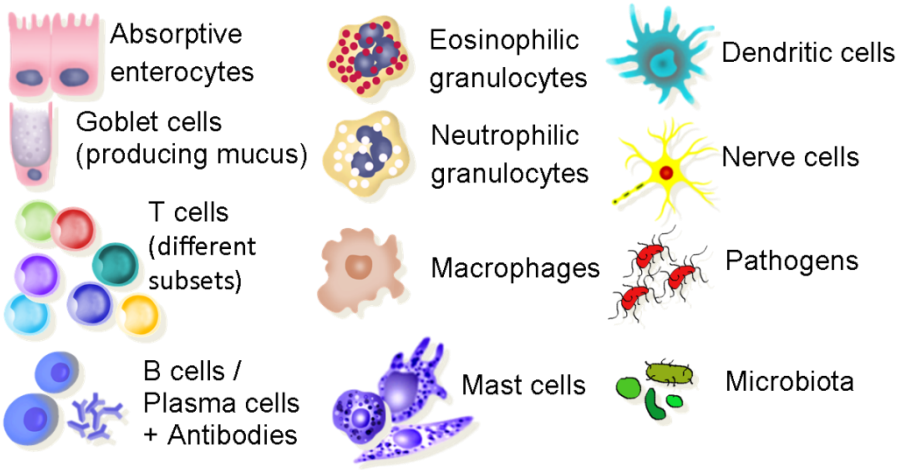


Figure 2.3-1: Scheme of the intestinal mucosal network in the small intestine (continued on next page)



Gut associated lymphoid tissue

(A) Diffusely in the mucosa located leukocytes (e.g. lamina propria lymphocytes or intraepithelial lymphocytes)

(B) Organised lymphoid tissue (aggregated lymphoid follicles)

Inductive site

Constant antigen sampling by

- (1a) - professional antigen presenting cells
- (1b) - normal villous epithelium
- (1c) - goblet cells
- (1d) - follicle associated epithelium

(2) Antigen processing/transfer in mucosa / associated lymph nodes

Effector site

Primed lymphocytes leave lymphoid tissue (via blood (3a) or lymph (3b)) and migrate back (4) to the intestinal mucosa

- (4a) - αβ T cells and B cells/plasma cells (lamina propria)
- (4b) - γδ T cells (epithelium)

Mucosal homeostasis and barrier function

Mast cells communicate locally with e.g.

(5a) - epithelial cells for e.g. regulation of mucus secretion

(5b) - blood vessels for e.g. control of blood flow and coagulation

(5c) - nerve elements (bidirectional communication) for e.g. smooth muscle contraction and peristalsis

(5d) - immune cells (attract leukocytes to sites of confrontation)

Mast cells communicate with targets in the distance:

(5e) - release TNFα containing particles

(5f) - particles enter lymphatics and travel to next draining lymph node

(5g) - lymph node enlargement is promoted

2.3.1. Gut associated lymphoid tissue - GALT

According to Köhne (1996), Rothkötter (2009) and Tizard (2013), a function of the GALT is either to induce IgA producing cells and regulatory T cells to establish a homeostatic relation with the microbiota and to avoid food allergies or to induce protective effector T cells. Therefore, the mucosa contains large amounts of lymphoid tissue (mucosa associated lymphoid tissue - MALT). In the gut, associated lymphoid tissue is either present as diffusely in the mucosa located leukocytes (e.g. lamina propria lymphocytes and intra-epithelial lymphocytes) (Figure 2.3-1/A) or as organized lymphoid tissue (groups of aggregated lymph follicles, like the Peyer's patches in the small intestine and lympho-glandular complexes in the large intestine) (Figure 2.3-1/B) (Köhne *et al*, 1996; Rothkötter, 2009; Tizard, 2013). Besides smaller discrete Peyer's patches throughout the small intestine, the pig has a large, continuous Peyer's patch in the last section of the distal small intestine. This contious Peyer's patch involutes within the first year of life (Rothkötter, 2009; Tizard, 2013). Components of the mentioned lymphoid tissues can be categorized into two functional groups, which mainly belong to the adaptive immune system. One is the inductive site, where antigens are processed and an appropriate immune response is initiated, and the other is the effector site, where antibodies and cell-mediated reactions are generated (Tizard, 2013).

Inductive site: According to Schulz (2013) and Tizard (2013), in order to balance the response against food proteins and mutualistic or potential harmful microbiota, the resident immune system is constantly sampling the intestinal content. Several mechanisms have developed for the transepithelial delivery of luminal antigen: Antigen presenting cells (dendritic cells and macrophages from the underlying lamina propria) can be recruited into the epithelium or extend paracellular processes through the epithelium scanning the gut epithelium and potentially the gut lumen (Figure 2.3-1/1a). The normal villous epithelium shuttles antigens by means of receptor mediated uptake of antigen–IgG complexes (Figure 2.3-1/1b). Also, goblet cells were recently shown to accumulate soluble antigen that dendritic cells process (Figure 2.3-1/1c). The epithelium, overlying lymphoid compartments (follicle-associated epithelium), is rich in microfold (M) cells that phagocytose and present particulate material to intraepithelial lymphocytes and dendritic cells or pass it on to the tissue fluid and underlying lymphoid tissue (Figure 2.3-1/1d). Antigen acquired via these mechanisms may be directly processed in the mucosa or in the mucosa associated lymphoid tissues and lymph nodes (Figure 2.3-1/2) (Schulz & Pabst, 2013; Tizard, 2013).

Effector site: After the immune response is initiated by antigen processing and presentation to B and T cells in the lymphoid tissue, primed lymphocytes leave the lymphoid tissue (Figure 2.3-1/3) and migrate back to the mucosa to exert effector functions (Figure 2.3-1/4) (Köhne *et al*, 1996). They do not necessarily migrate back to the intestinal mucosa. IgA-producing B cells can migrate into regional lymph nodes, enter the circulation and settle e.g. in the mammary

gland, which ensures that milk contains IgA antibodies directed against intestinal pathogens. Also, T cells which were primed in the GALT and preferably home to the intestinal mucosa can be found in other tissues like the mammary gland. To reach their destination, these B and T cells use special adhesion molecules expressed on vessels in the intestinal lamina propria as well as the mammary gland (Tizard, 2013). Effector T cell subsets prefer different locations within the mucosa. Porcine αβ T cells are mainly found in lymphoid tissues and in the lamina propria of villus and crypt (Figure 2.3-1/4a), whereas γδ T cells are predominantly located in the intestinal epithelium (Figure 2.3-1/4b). These intraepithelial lymphocytes are a heterogeneous population of cells with a diverse array of functions in epithelial surveillance. They may present antigens, regulate B cell IgA responses, have natural killer cell properties, are cytotoxic T cells or take part in repair of damaged epithelia and thus are an important element in mucosal homeostasis and barrier function (Charerntantanakul & Roth, 2006; Tizard, 2013).

2.3.2. Mucosal homeostasis and barrier function

The central function of the intestinal tract is water, electrolyte and nutrient distribution to sustain the organism. As this comes with an intimate contact to the outside world and a high bacterial and antigen load, the gut mucosa forms a selectively permeable barrier that is tightly regulated and actively participates in host defence through the mucosal immune system (Shen, 2009).

According to Shaykhiev and Bals (2007), who investigated interactions between epithelial cells and leukocytes in the mucosal network, the epithelium is the linking as well as the separating element between luminal content (“the outside world”) and the body interior.

Besides the crucial function in nutrient digestion and absorption, the epithelium provides the separating physical barrier to keep out microorganisms. Key aspects of this physical barrier are cell polarity, secretion of mucus and antimicrobial substances and a strict separation of the luminal and basolateral cell compartment via tight junctions (Shaykhiev & Bals, 2007). The basolateral space of the epithelial cells is connected to the underlying lamina propria via pores in the basement membrane. Intercellular fluid passes through these pores to mix with that in the intercellular spaces and prelymphatic intercellular channels of the lamina propria. From there, lymph enters lacteals in the villi, or the network of blood vessels within the lamina propria (Lowden & Heath, 1994).

The linking function of the epithelium includes antigen recognition via pattern recognition receptors and antigen presentation (see 2.3.1). Wells et al. (2010) describe that intestinal epithelial cells express a range of pattern recognition receptors to sense the presence of microbes. This process plays a key role in pathogen recognition and the induction of innate effectors and inflammation. Several signalling adaptations have evolved to avoid uncontrolled

inflammatory responses toward the resident microbiota (Wells *et al*, 2010). The epithelium also has additional immunosuppressive functions. Epithelial secreted cytokines inhibit overreaction against inoffensive luminal antigens by regulation of dendritic cells, macrophages and lymphocytes (Wells *et al*, 2010). A diverse lymphocyte population directly inhabiting the epithelium are the already mentioned intraepithelial lymphocytes. Many of them are highly sensitive and fully differentiated antigen-experienced memory T-cells, which function as effective sentinels and rapidly respond to pathogens (Cheroutre & Huang, 2012). They also monitor the epithelium for signs of damage, recruit inflammatory cells to the site of damage and directly mediate epithelial repair via secretion of epithelial growth factors, e.g. keratinocyte growth factor (Havran *et al*, 2005; Meehan *et al*, 2014) thus keeping up the epithelial barrier. Interestingly, keratinocyte growth factor also protects endothelial barrier function and induces angiogenesis in the microvasculature (Gillis *et al*, 1999).

A dynamic cross-talk is going on between the intestinal epithelial cells and the lymphocyte-rich lamina propria. The enterocytes have the capacity to activate populations of unique regulatory T cells. In return, these cells have the capacity to influence epithelial cell growth and differentiation (Dahan *et al*, 2007). Regulatory cells can modulate immune responses by direct suppression of specific subsets of effector cells, or by redirecting effectors against invading pathogens. Classically, these functions are carried out by different T cell subsets. Additionally, mast cells have been shown to regulate physiological and pathological immune responses (Frossi *et al*, 2010). According to Van Nassauw et al. (2007) mast cells have a central position in the intestinal mucosal network consisting of the epithelial cells, immune cells, blood vessels, smooth muscle and intramurally located nerve elements, since they are able to detect noxious and antigenic threats and to generate or amplify signals to the other cells. A bidirectional communication seems to exist between neurons and mast cells (Figure 2.3-1/5c) within the gastrointestinal tract (Van Nassauw *et al*, 2007). As described by Bischoff (2009), mast cells account for 2-3% of lamina propria cells and are generally associated with allergic disease, although they have a greater number of physiological functions. They regulate functions such as control of blood flow and coagulation (Figure 2.3-1/5b), smooth muscle contraction and peristalsis (Figure 2.3-1/5c), and secretion of acid, electrolytes, and mucus (Figure 2.3-1/5a) by epithelial cells. Furthermore, they have defence mechanisms combining innate and adaptive immune responses against pathogens and possibly the commensal flora. They recognize microbes by antibody-dependent mechanisms and through pattern-recognition receptors. Upon activation, they secrete cytokines, which attract granulocytes and lymphocytes to the site of confrontation (Bischoff, 2009). In addition to a local effect, mast cell signals also reach targets in the distance. Recently, it was shown very elegantly by Kunder et al (2009) that, upon activation, mast cells release stable submicrometer heparin-based particles containing TNFα (tumor necrosis factor alpha), a cytokine involved in systemic

inflammation via modulation of immune cell trafficking (Figure 2.3-1/5e). These particles enter the lymphatic system and travel to the next draining lymph node (Figure 2.3-1/5f) where they promote lymph node enlargement (Figure 2.3-1/5g). This is an important process, where the lymph node quickly undergoes significant structural changes, including rapid cellular proliferation, vascular remodelling and enhanced recruitment as well as retention of naive lymphocytes from the circulation. Via this process, the probability that an activated antigen presenting cell from the site of pathogen entry meets lymphocytes bearing a fitting receptor is greatly enhanced and thus a critical initiating event in the development of the adaptive immune response (Kunder *et al*, 2009). Another interesting physiological function of mast cells is their regulation of intestinal epithelial migration. It was possible to demonstrate that mice deficient in mast cells had significantly decreased basal small intestinal permeability, which was linked to decreased intestinal epithelial cell migration along the villus/crypt axis as well as altered intestinal morphology and barrier function (Groschwitz *et al*, 2009).

The resident immune system so far described is not the only component necessary for maintaining intestinal homeostasis and regulation of intestinal epithelial barrier function. As already briefly mentioned, the intestinal flora also plays an important role.

2.3.3. Relationship of intestinal microbiota, pathogens and the host

The collection of microorganisms (bacteria, archaea, viruses, unicellular eukaryotes) which live in peaceful coexistence with their host is referred to as microbiota. The composition of the gut microbiota is host specific and may be altered via internal as well as external factors during the course of an organism's lifecycle (Sekirov *et al*, 2010).

From an evolutionary point of view, host-bacteria relationships in the gastrointestinal ecosystem have developed to the mutual benefit of both partners. The gastrointestinal tract of the host provides an attractive habitat for bacteria (and other microorganisms), granting nutrients and space for survival. In return, the microbiota is involved in various physiological functions, e.g. regulation of intestinal epithelial cell turnover, promotion of epithelial repair and reorganization of tight junctions. Both parties collaborate as long as possible to each others profit, pause this cooperation in case of a crisis and during this, or afterwards, work to re-establish intestinal homeostasis and cooperation. In this context, the mucosal epithelium not only physically separates the microbiota from the "rest of the body", but also serves as a sentinel to the underlying immune cells (Chow *et al*, 2010; Yu *et al*, 2012).

Since bacteria and animals have a common evolutionary heritage and many animal genes are homologs of bacterial genes, they are able to recognize one another and share information (McFall-Ngai *et al*, 2013). As an example, signalling by the microbiota through toll-like receptors of the host seems to be important for maintaining intestinal epithelial homeostasis (Chow *et al*, 2010). Thus, the microbiota can communicate with the host, and vice versa the

host can send signals to the microbiota and additionally to pathogens. Different microbiota species interact with each other and with pathogens and of course, pathogens interact with the microbiota, the host and with each other (Sekirov *et al*, 2010; Singer, 2010).

Not only microbiota and host share a common co-evolutionary history. Pathogens developed simultaneously and acquired counter strategies to “hijack” cellular molecules and signalling pathways of the host and its microbiota (Srikanth & McCormick, 2008). E.g. *Salmonella* Typhimurium senses the host hormones epinephrine and/or norepinephrine and in response upregulates virulence factors, involved in epithelial invasion (Moreira & Sperandio, 2012; McFall-Ngai *et al*, 2013). Pathogens have developed intricate concepts to invade the mucosal epithelium, corrupting barrier integrity and promoting systemic spreading. The pathogens may take advantage of an already existing barrier breach or weakness as well as actively cross the barrier by themselves. They translocate across the epithelium via different routes, e.g. by disrupting tight junctions and taking a paracellular way, or they may also travel through absorptive enterocytes, dendritic cells and M cells (Doran *et al*, 2013).

The detection of pathogens by the immune mechanisms of the host, breaching the host’s barrier and colonizing the lamina propria, results in the induction of a strong inflammatory response to control the pathogen. This response involves cytokine release, neutrophil recruitment and release of antimicrobial substances and also reduces the viability of the microbiota - which turns out to be advantageous for the pathogen as it will occupy free niches. For some pathogens like *Salmonella* Typhimurium, the induction of inflammation has even been shown to be necessary. To overcome colonization resistance, inflammation provided a shift in the balance between the protective microbiota and the pathogen in favour of the pathogen (Stecher *et al*, 2007; Sekirov *et al*, 2010).

To reduce the incidence of inflammatory, infectious and autoimmune diseases, early microbial colonization of the gut was shown to be important, supporting the ‘hygiene hypothesis’ (Mulder *et al*, 2009), which i.a. implies that reduced microbial contact is a key factor in the ongoing rise of hypersensitivity reactions (Bloomfield *et al*, 2006). In humans, a microbiota dominated by lactobacilli may function to maintain mucosal immune homeostasis and limit pathogen colonization (Mulder *et al*, 2009). As hundreds of bacterial species make up the mammalian intestinal microbiota, a change in this ecosystem, caused by antibiotics, diet, immune deficiency or infection, may lead to a loss of balance - a dysbiosis. This can result in overgrowth (blooming) of otherwise under-represented or potentially harmful bacteria. Additionally, dysbiosis may stimulate the transfer of virulence and antibiotic resistance genes and thereby supports pathogen evolution (Stecher *et al*, 2013).

2.4. Nutritional influences on microbiota, pathogens and host

The composition of the gut microbiota and its metabolite production are strongly influenced by diet and in return, can have a major influence on metabolic disorders like obesity and diabetes in humans. The astonishing amount of one third of an animal's metabolome (the pool of chemical compounds involved in an organism's metabolism) found in the blood circulation has microbial origin. Via this way, the whole organism can be influenced by the resident gut microbiota (McFall-Ngai *et al*, 2013).

An area under discussion around the critical time of weaning is how the respective feed should be composed. In addition to poor growth and development associated with reduced feed intake after weaning, young pigs are immunologically immature and thus especially prone to diarrhoea caused by enteric pathogens. They e.g. have an increased risk for disease through infection with enterotoxigenic *Escherichia coli* and *Salmonella* (Lallès *et al*, 2007).

Therefore, the aim of an optimum nutrition around weaning is not only a good feed acceptance, but also the potential reduction of pathogens and support of the developing microbiota.

Feeding strategies like a special diet composition and/or feed additives such as probiotics have been shown to be effective in reducing pathogenic bacteria, promoting animal growth and supporting the microbiota (Pluske, 2013).

2.4.1. Probiotics as feed additives

Based on the concept that resident microbiota and host live in a beneficial symbiosis, modulation of the microbial community via probiotics has been explored as a prevention or treatment of disease (Ruemmele *et al*, 2009). Probiotics are used with the intention to make the immune system work more efficiently (Bailey, 2009). They may promote mucosa-bacteria interactions via the secretion of extracellular proteins (Sanchez *et al*, 2010); modulate the establishment of lymphocyte populations and IgA secretion in the gut and reduce bacterial translocation to mesenteric lymph nodes (Lessard *et al*, 2009); favourably influence piglet performance and intestinal morphology (Di Giancamillo *et al*, 2008).

The probiotic bacterial strain used in our studies was *Enterococcus faecium* NCIMB 10415 (*E. faecium*), which belongs to the enterococci. Several species belong to this genus and they in turn belong to the lactic acid bacteria. *E. faecium* is a frequently used commercial feed additive (Cylactin®) in pig nutrition. Different reports about the efficacy of *E. faecium* are present in the literature. They range from no effect at all over a positive influence on weight gain and feed conversion ratio to a protection from pathogens. Also a reduced resistance to pathogens was observed (Pollmann *et al*, 2005; Broom *et al*, 2006; Reiter *et al*, 2006; Szabo *et al*, 2009; Mallo *et al*, 2010; Bednorz *et al*, 2013; Siepert *et al*, 2014). Franz et al (2009) report that, besides their use as probiotics, enterococci are involved in food spoilage, fermentation

and nosocomial infections. Their role in disease has raised questions on their safety for use in foods or as probiotics. Therefore the potential of gene transfer in the gastrointestinal tract was also described to be of concern when using enterococcal probiotics. The traits (positive as well as negative) are highliy strain specific. The strain used in this study is one of the best characterised in terms of safety and has been used safely for over 20 years (Franz *et al*, 2011).

2.5. Histological techniques applied to investigate the gastrointestinal tract

Histological techniques (histochemistry and immunohistochemistry) are valuable tools in biomedical sciences with a long tradition (Coleman, 2000; Wick, 2012). They provide information on the effects different treatments have on a variety of parameters. For example changes in diet, the microbiota or pathogen challenges can alter the histochemical composition of mucosubstances, the intestinal microanatomical morphology or the mucosal immunity (Sharma *et al*, 1995; Cruz Sanchez *et al*, 2008; Che *et al*, 2009). Histology may even be superior to other techniques in identifying and characterizing cells and microorganisms. In a study about *Erysipelothrix rhusiopathiae,* an immunohistochemical assay was shown to be especially effective for its rapid detection of the pathogen in comparison with direct cultures of organs (Opriessnig *et al*, 2010).

There exists a considerable heterogeneity between animal models used and between experimental settings employed by different researchers. Performing immunohistochemistry can be challenging because several epitopes react to fixation and embedding in a species-specific manner. Therefore it is necessary to constantly adapt and improve histological techniques (Pellicciari, 2013; Scalia *et al*, 2015).

2.6. Nutrition-microbiota-pathogen-host-interactions: A graphic abstract

Figure 2.6-1 gives an overview, summarizing the main findings and functional relationships between nutrition, microbiota, pathogens and the host from the literature review.

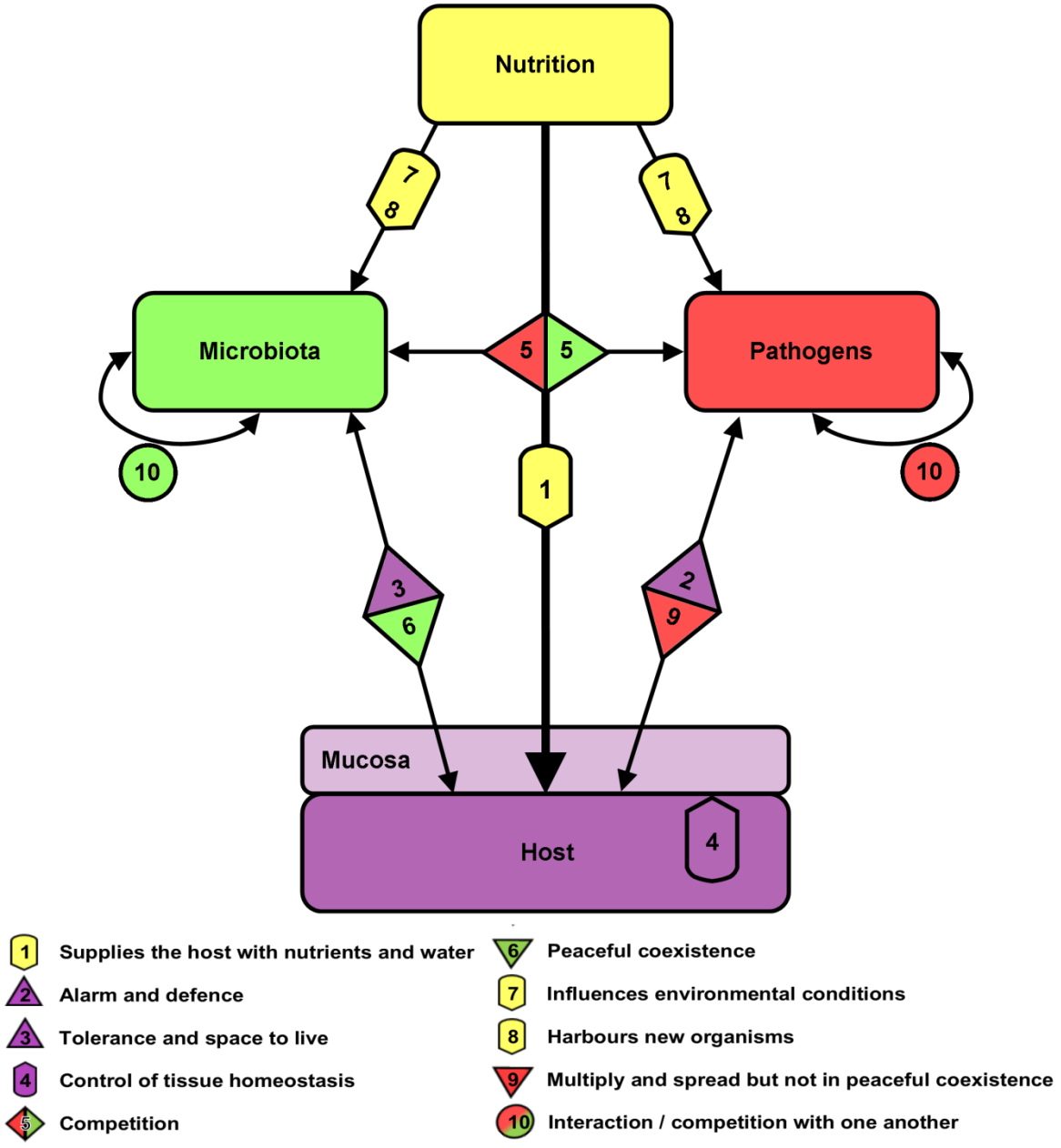


Figure 2.6-1: Interactions between nutrition – microbiota – pathogens and host on the intestinal level. Overview, summarizing the findings from the literature review.

3. Aims and objectives of the thesis

There are various literature reports on the efficacy of the frequently-used probiotic *E. faecium*. They range from no effect at all over a positive influence on performance (e.g. weight gain or feed conversion ratio) and protection from pathogens to reduced resistance to pathogens. Since the intestine is the interface where nutrition, microbiota, pathogens and host meet, the objective of the thesis was to further our knowledge on the effect of *E. faecium* in a *Salmonella* challenge situation, with particular focus on critically involved components of the intestinal mucosal network.

It is hypothesised that favourable effects of *E. faecium* treatment under a *Salmonella* challenge involve beneficial changes in performance and immunologically related parameters of the intestinal mucosal network.

- As an indicator for enhanced performance, the mucosal surface available for nutrient absorption is expected to be enlarged.
- As an indicator for improved immune protection, epithelial barriers defending or regenerating intraepithelial lymphocyte populations are expected to be increased.
- As an additional indicator for improved immune protection, the number of bacteria (*Salmonella*) invading the mucosa is expected to be lower.

Since mast cells were identified as a central cell population with a multitude of physiological and pathological functions, including regulation of intestinal barrier function and host defence within the intestinal mucosal network, it is hypothesised that these cells are influenced by probiotic and other treatments in the SFB 852 trials.

It is hypothesised that (species-) specific histological protocols will improve the detection of pathogens and identification of immune cells within porcine tissues.

- The first study was planned in the course of a feeding trial, in which the performance of probiotic-treated (*Enterococcus faecium* NCIMB 10415), weaned piglets was to be observed in a challenge situation with *Salmonella* Typhimurium. Therefore, the jejunal intestinal mucosal surface available for nutrient absorption was to be determined morphometrically. As an immunologically important component of the epithelial barrier, the intraepithelial lymphocytes were to be quantified *in situ* with histochemical methods and characterized via immunohistochemistry.

- The second study focussed on the immunohistochemical detection of *Salmonella* Typhimurium in porcine mucosa-associated tissues to visualize its *in situ*

localization in addition to e.g. bacterial quantification results of our working group for homogenized tissues (published by Kreuzer *et al*, 2012).

- The third study was to focus on porcine intestinal mast cells. A species specific protocol was to be established to reliably quantify mast cells in porcine tissues and find a basal cell number for further investigations in other SFB 852 trials. Histochemical as well as immunohistochemical methods were to be used.

4. Intraepithelial lymphocyte numbers and histomorphological parameters in the porcine gut after *Enterococcus faecium* NCIMB 10415 feeding in a *Salmonella* Typhimurium challenge

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Intraepithelial lymphocyte numbers and histomorphological parameters in the porcine gut after *Enterococcus faecium* NCIMB 10415 feeding in a *Salmonella* Typhimurium challenge

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Abstract

Salmonellae are among the most widespread sources of foodborne infections and *Salmonella* Typhimurium, in particular, is correlated with human disease caused by the consumption of contaminated pork. Intraepithelial lymphocytes (IEL) have early contact with intestinal antigens and play an important role in the detection of pathogenic bacteria. The objective of this study was to determine whether a presumed probiotic *Enterococcus faecium* strain could improve histomorphological and immune system-related parameters of gut function after a *Salmonella* challenge in weaned pigs. In particular the morphological parameters villus length and width, crypt depth and width as well as the actual enlargement of

the intestinal epithelial surface were calculated and the number of IEL was evaluated in sections of the porcine gut.

Weaned piglets were challenged with *Salmonella enterica* serovar Typhimurium DT 104, and half of them also received *Enterococcus faecium* NCIMB 10415 in the diet. Animals were sacrificed at days post infection (DPI) 2 and 28. The effect of the factors “time post-infection/age” and “probiotic treatment” on jejunal morphology and IEL numbers and distribution was evaluated by light microscopy. The time post-infection had significant effects in both feeding groups. Animals sacrificed at DPI 28 had longer and wider villi, deeper and wider crypts, a higher villus enlargement factor, a higher ratio between villus and crypt enlargement factors as well as more IEL. Probiotic treatment resulted in longer villi, a higher ratio of villus surface/crypt circumference enlargement factors and significantly more IEL. The larger total number of IEL displayed by the probiotic group resulted from significantly higher numbers of IEL at the nuclear and apical levels of the intraepithelial compartment but not from the number of IEL situated at the basement membrane. The probiotic effects were only measurable 28 DPI. It is proposed that *Enterococcus faecium* NCIMB 10415 exerts an immune modulatory effect by increasing the numbers of intraepithelial lymphocytes.

Keywords

Intraepithelial lymphocytes; Mucosal immunity; Probiotics; *Salmonella*; Swine

Abbreviations

IEL, intraepithelial lymphocyte(s); *S.*Typhimurium, *Salmonella enterica* serovar Typhimurium; *E. faecium*, *Enterococcus faecium*; DPI, days post infection; CFU, colony forming units

4.1. Introduction and aim

Salmonellae are among the most widespread sources of foodborne infections and especially *Salmonella enterica* serovar Typhimurium (*S.*Typhimurium) that often contaminates pork (Hedemann et al., 2005, Kreuzer et al., 2012 and Martins et al., 2012). In pigs, *S.*Typhimurium infection causes clinical symptoms with enterocolitis and regularly subclinical infections persist. These infected animals can act as a host reservoir and bring the pathogen into the food chain (Boyen et al., 2008 and Collado-Romero et al., 2012). *Salmonella* infects epithelial cells of the small and large intestine and may cross this barrier via different mechanisms (Hulst et al., 2013, Schauser et al., 2004 and Velge et al., 2012). It is crucial that mammalian hosts monitor and regulate microbial interactions with intestinal epithelial surfaces (Duerkop et al., 2009). Intestinal intraepithelial lymphocytes (IEL) are the first in line to have contact with the antigen present in the gut (Hershberg and Blumberg, 2005) and belong to a unique T-cell population (Chang et al., 2005). They are located between the enterocytes in the epithelium above the basement membrane and are phenotypically and functionally distinct from lymphocytes in the underlying lamina propria or draining lymph nodes (Lacković et al., 1999, Vega-López et al., 1993, Waly et al., 2001 and Whary et al., 1995). IEL play an important role in the detection of pathogenic bacteria, are involved in the discrimination between pathogenic and commensal organisms and have been demonstrated to increase in numbers after oral infection by *S.*Typhimurium (Li et al., 2012). Under homeostatic conditions, IEL regulate the continuous turnover of epithelial cells by the eradication of infected epithelial cells and the control of epithelial repair (van Wijk and Cheroutre, 2009). The proliferation and apoptosis of the surface epithelium may also be influenced by probiotics which are claimed to improve intestinal health of pigs, reduce mortality and increase productivity rates (Cho et al., 2011 and Resta-Lenert and Barrett, 2003). Probiotics are dietary additives, recognised to provide beneficial effects to the gastrointestinal tract, for example by modulating gut morphological features as well as the gut's immune system including intestinal lymphocytes (Baum et al., 2002 and Roselli et al., 2009).

This study is part of a wider group of research activities with the aim to understand how nutritional factors influence the functioning of the intestines in the pig. Previous to the herein described challenge trial, several feeding experiments with a presumed probiotic *Enterococcus (E.) faecium* strain were carried out by our Collaborative Research Centre (SFB 852 “Nutrition and intestinal microbiota – host interactions in the pig” http://www.sfb852.de) and an earlier Research Unit (FOR 438). In different trials with pigs, a variety of observations were made. For example, in unchallenged piglets, *E. faecium* has been found to increase the absorptive and secretory capacity of jejunal mucosa (Klingspor et al., 2013) and reduce levels of cytotoxic T cells in the jejunal epithelium (Scharek et al., 2005), while mucosal morphology, villus height,

crypt depth and the enlargement factor were not influenced (Martin et al., 2012 and Reiter et al., 2006).

The objective of this study was to determine whether *E. faecium* could influence histomorphological parameters of gut function as well as IEL number and distribution in combination with a *Salmonella* challenge in weaned pigs. Because the results reported in this paper are part of a larger trial, several other parameters from the same experiment have already been reported in earlier publications. The following summarises these. Kreuzer et al. (2012) report that piglets treated with *E. faecium* gained less weight and that they had a higher incidence of the pathogen in tonsils. Conversely, *E. faecium* had no effect on fecal shedding of *Salmonella*. The specific (anti-*Salmonella* IgG) and nonspecific (haptoglobin) humoral immune responses as well as the cellular immune response (T helper cells, cytotoxic T cells, regulatory T cells, γδ T cells and B cells) in lymph nodes, Peyer's patches, the ileal papilla and in the blood do not seem to be affected by *E. faecium* treatment (Kreuzer et al., 2012). However, when analysing magnetically sorted lymphocytes from ileocecal mesenteric lymph nodes, differences in the activation of T helper cells between the treatment groups were found. It was concluded that a higher Th2 driven immune response in the control group could have been the reason for the better *Salmonella* clearance reported for the control group in this as well as in an earlier challenge study (Kreuzer et al., 2014). Siepert et al. (2014) have given a comprehensive summary of our previous and latest feeding experiments concerning several immunological aspects. They conclude that *E. faecium* may have an anti-inflammatory/immuno-suppressive effect in piglets, which becomes most apparent during the post-weaning period (Siepert et al., 2014).

4.2. Materials and methods

4.2.1. Study design and probiotic feeding in the *Salmonella* challenge trial

The experimental setup has already been described (Kreuzer et al., 2012). In short, sows of the German Landrace breed were fed daily either a diet supplemented with 4.3×10^6 colony forming units (CFU) of *E. faecium* NCIMB 10415/g feed from day 28 ante partum onward or with the control diet containing no *E. faecium. E. faecium* NCIMB 10415 is a commercial probiotic feed additive (Cylactin® LBC ME10, DSM Nutritional Products Ltd, Switzerland). Piglets of the probiotic group ($n = 16$) were offered creep feed supplemented with 5.1×10^6 CFU/g in the suckling period and 3.6×10^6 CFU/g post weaning (d 28). Piglets from the control group ($n = 16$) received the same feed without the probiotic. Details of the ingredients and chemical composition of the diets were the same as reported (Martin et al., 2012).

The piglets were allocated to boxes in pairs. At the age of 38 days all piglets were challenged intragastrically with *S. enterica* serovar Typhimurium DT104 (1.4–2.1 × 10^{10} CFU) using a stomach tube. Six piglets from each group were sacrificed at DPI 2 and ten piglets on DPI 28, respectively. The rationale behind the time points of sacrificing the piglets is based on earlier studies of our working group (Mafamane, 2009 and Szabó, 2009). In summary, between 24 and 48 h post infection most animals show clinical symptoms such as fever and diarrhoea. The late sampling date (28 days post infection) was chosen to examine the long time effects of probiotic feeding and *Salmonella* infection. The animals were infected 10 days later than in the first experiment (Szabó, 2009 and Mafamane, 2009) because of a newly implemented adaptation period to the new environment. It should be noted that all animals in our study were challenged with *Salmonella* and so the "control group" in our study also includes infected animals. This definition of the term "control group" in animal infection studies was established earlier (Johnson and Besselsen, 2002 and Sargeant et al., 2014). Experimental approval was given by the local authority (Landesamt für Gesundheit und Soziales, Berlin ID: G0348/09).

4.2.2. Sample collection and histochemical staining

Samples of mid-jejunum, ileum, colon ascendens and tonsilla veli palatini were taken immediately after slaughter and rinsed in ice cooled Ringer's solution. Gut samples were cut open on the mesenterial side, trimmed to 2 cm × 1 cm squares and pinned mucosal side up on cork pieces. Fixation of all samples was done for 26 h in Zamboni's fixation solution. Fixed tissues were processed according to standard histological protocols and stained with H&E or panoptic Pappenheim tissue stains (Romeis, 1989).

4.2.3. Morphometric parameters assessed by light microscopy

For each sample at each point in time, 4 slides were prepared and morphometric parameters were evaluated under a light microscope (Axioplan, Carl Zeiss, Jena, Germany). Digitalised live pictures were analysed on a monitor with the help of the computer assisted image analysis program NIS-Elements AR (Nikon Instruments Inc., U.S.A.). The following parameters were determined as previously described (Wiese, 2003 and Wiese et al., 2003):

- villus length
- villus width
- crypt depth
- crypt width
- length of villus surface (equalling the length of the enterocyte brush border)
- total circumference of crypts demarcated by their basement membrane
- corresponding length of the lamina muscularis mucosae in the area measured

Measurement was only done in well oriented parts of the sections, which means at least 4 villi were cut completely from tip to base and most crypts were cut longitudinally. Supplemental Fig. 1A gives an overview on how the parameters were measured. A minimum of 5 high power fields (total magnification ×50) were measured. To estimate the enlargement of the intestinal surface epithelium by villi and crypts, the villus and crypt enlargement factors were calculated as follows:

$$Villus\ enlargement\ factor\ = \frac{length\ of\ the\ villus\ surface}{length\ of\ the\ corresponding\ lamina\ muscularis\ mucosae}$$

$$Crypt\ enlargement\ factor\ = \frac{summed\ up\ crypt\ circumferences}{length\ of\ the\ corresponding\ lamina\ muscularis\ mucosae}$$

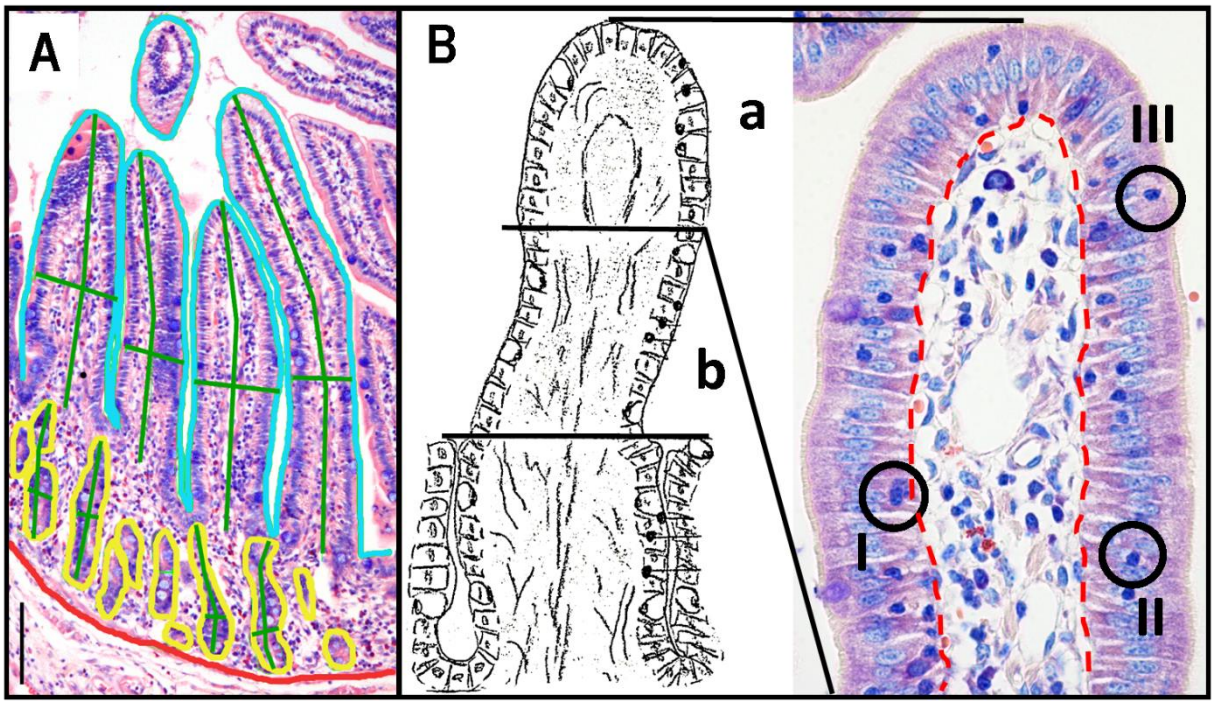


Figure 4.2-1: Supplemental figure 1 - Illustration of the measurement procedures:

A) Morphometric parameters like villus length and width, crypt depth and crypt width are represented with dark green lines. The length of villus surface (resembling the length of the enterocyte brush border) is indicated in light blue and the total circumference of crypts demarcated by their basement membrane in yellow. The corresponding length of the lamina muscularis mucosae in the area measured is marked in red.

B) The number and distribution of jejunal IEL along the villi was registered separately for tip (a) and base (b) of villi. Basal (I), peri-nuclear (II) and apical (III) intraepithelial compartments were distinguished. The corresponding enterocyte basement membrane (red dashed line) was measured so that the number of IEL could be calculated as IEL/100 µm basement membrane. IEL were identified by their morphology i.e. small lymphocytes with a round and intensely stained nucleus and little cytoplasm or larger lymphocytes with distinct cytoplasm located in the epithelium between enterocytes.

4.2.4. Counting of IEL by light microscopy

The number and distribution of jejunal IEL along the villi was evaluated within three different intraepithelial compartments, namely the apical, the perinuclear and the basal compartments as described elsewhere (Vega-López et al., 2001; see also Supplemental Fig. 1B). In brief, counting was done using a 400× magnification, centering a randomly chosen, well-orientated villus tip or villus base in the field of view. In case of short villi (tip and base overlapping in one field of view) only the tip was evaluated. In each sample 10 villi were analysed. IEL were identified by their morphology i.e. small lymphocytes (7–10 µm in diameter) with a round and intensely stained nucleus and little cytoplasm or larger lymphocytes (10–20 µm in diameter) with distinct cytoplasm located in the epithelium between enterocytes (Stokes et al., 1994, Vega-López et al., 2001 and Wilson et al., 1986). As IEL were counted, the length of the corresponding enterocyte basement membrane was measured and finally the number of IEL could be calculated as IEL/100 µm basement membrane. The number was registered separately for tip and base of villi.

4.2.5. Statistical analysis

Statistical evaluations were carried out using the software package IBM SPSS 21 (IBM Deutschland GmbH, Ehningen, Germany). All variables were tested by two-way ANOVA with the factors “Age” (time post infection) and “Group” (feeding group). Since for some parameters age and feeding groups interacted significantly, we analysed each age group separately using the *t*-test for independent samples. IEL measurements at different localisations but from the same sample were compared by the *t*-test for paired samples. In order to achieve normality and homoscedasticity of the data expressed in relative frequencies (percentages), these were square root-arcsine transformed before parametric testing. However, for the convenience of the reader we report means of the untransformed data. The level of significance was $\alpha = 0.05$. If groups were analysed separately or paired *t*-tests within groups were performed, the level of significance was adjusted to $\alpha = 0.025$ (Bonferroni adjustment). Results from separately analysed groups reaching a significance level between $\alpha \leq 0.05$ and $\alpha > 0.025$ were considered to be tendentially significant.

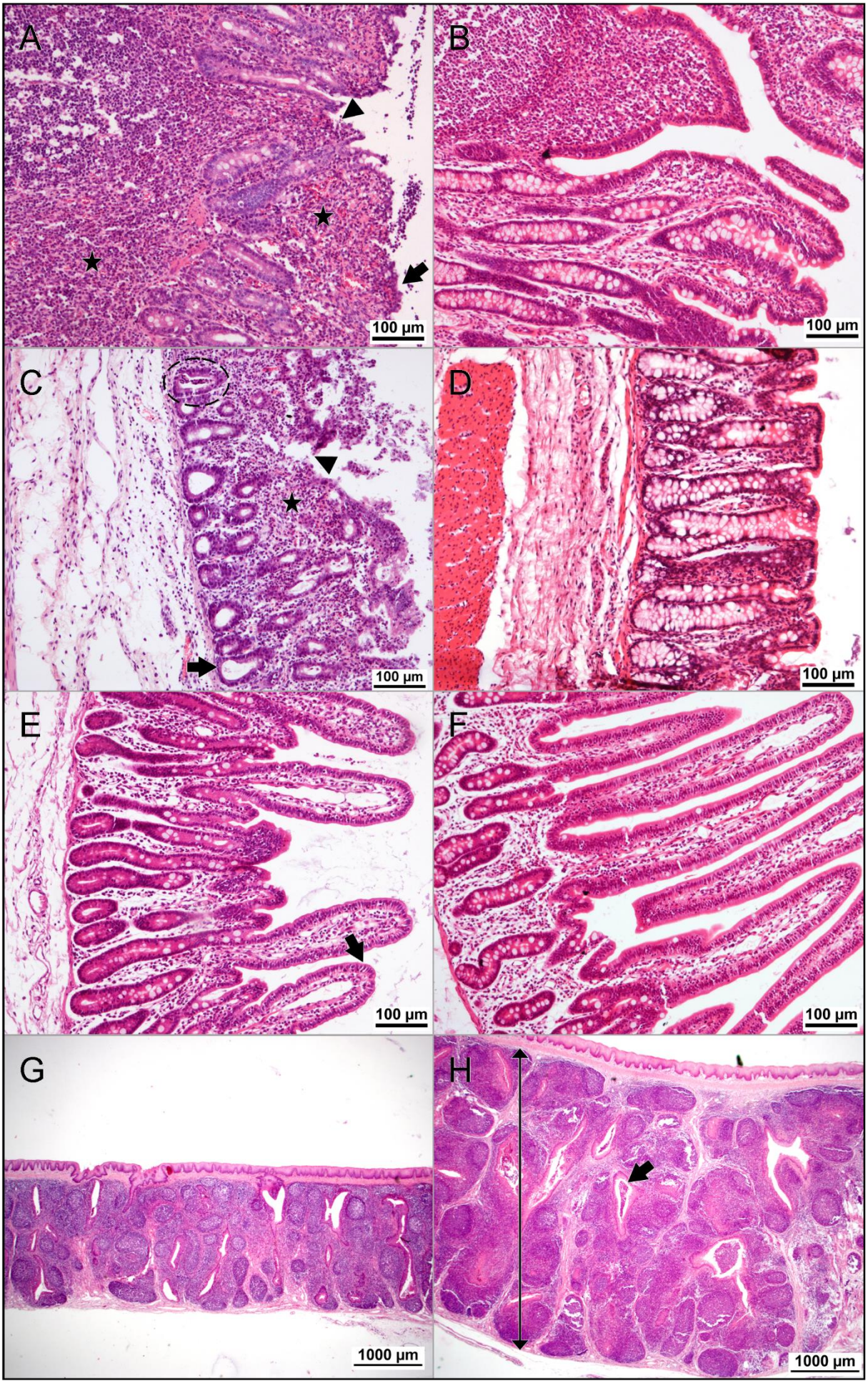
4.3. Results

4.3.1. Pathological findings

*S.*Typhimurium provoked damage of the intestinal mucosa, which was seen clearly in the ileum and colon. At DPI 2, villus atrophy was found with varying degrees of severity in the jejunum and ileum, as already described in our previous publication (Kreuzer et al., 2014). Neutrophil granulocyte infiltration, erythrocyte extravasation, as well as flattening, disintegration and degeneration of the surface epithelial layer plus the accumulation of cell detritus were found in the ileum, colon and occasionally in the jejunum of animals at DPI 2 (Fig. 1A, C and E). At DPI 28, the intestinal mucosa was found to recover with largely normal morphology (Fig. 1B, D and F). Tonsils of animals at DPI 2 exhibited follicular hyperplasia. Leukocytes, especially neutrophil granulocytes, were present in high numbers. The crypt epithelium was infiltrated with leukocytes, which were also present in the crypt lumen. Tonsils from DPI 28 animals were around 50% larger than those from DPI 2 animals. The enlargement was mainly attributed to the B cell areas where high densities of inflammatory cells were noticeable. The crypt epithelium was damaged and infiltrated by leukocytes. Crypts were filled with desquamated epithelium and inflammatory cells, mainly neutrophils (Fig. 1G and H).

Figure 4.3-1: Figure 1 - Pathohistology: ►►►

Pictures on the left side of the panel show H&E stained tissue sections of ileum (A), colon ascendens (C), jejunum (E) and tonsilla veli palatini (G) of animals 2 days post infection (2 DPI). The corresponding pictures on the right side (B, D, F, and H) represent tissues from animals 28 DPI. Differing degrees of *Salmonella* induced damage can be identified. A) In the ileum villus atrophy up to a complete loss of villi (arrow), epithelial erosion (arrowhead) and massive inflammatory cell infiltration (asterisk) is noticeable. B) Ileal tissue structure at 28 DPI has a normal appearance. C) The example from the colon at 2 DPI shows a region with mucosal erosion (arrowhead). The lamina propria is markedly expanded due to infiltration with numerous inflammatory cells (asterisk). There are crypt abscesses (encircled), the crypts are lined by flattened epithelial cells (arrow) and only few goblet cells are visible in comparison with the corresponding picture (D). E) The jejunum at 2 DPI shows atrophic villi (arrow) which had regenerated fully by 28 DPI (F). G and H) At day 28 post infection an increased thickness of the parenchyma, attributed to the tonsillar follicles and crypts (double arrow) which are filled with cellular detritus (arrow), is noticeable.


A
100 µm
B
100 µm
C
100 µm
D
100 µm
E
100 µm
F
100 µm
G
1000 µm
H
1000 µm

4.3.2. Morphometric parameters and IEL counts assessed by light microscopy

The time post infection (age) had significant effects ($P < 0.05$) on most morphometric parameters and IEL numbers. Older animals (28 DPI) had longer and wider villi, deeper and wider crypts, higher villus enlargement factor, higher ratio between villus and crypt enlargement factors as well as more IEL (Table 1).

Feeding *E. faecium* had overall significant effects ($P < 0.05$) on IEL numbers, the probiotic group generally having higher numbers (Table 1). Analysing age groups separately by *t*-test showed that *E. faecium* had measurable effects only on parameters determined after day 28 PI: Probiotic fed animals tended to have longer villi ($P = 0.037$) and a higher ratio of villus surface/crypt circumference enlargement factors ($P = 0.046$) (Supplemental Fig. 2A–E and Table 1). Significantly more IEL ($P < 0.025$) were present in the jejunal epithelium of animals treated with *E. faecium*. The IEL located at the nuclear level of the intestinal epithelium presented the most prominent quantitative differences (Supplemental Fig. 2F–I and Table 1).

The distribution of IEL within the epithelium was uneven ($P < 0.001$). Over all age and feeding groups, 60% of IEL were found in the basal region, 27% in the nuclear region and 12% in the apical region of the mucosal epithelium. We were able to detect significant differences ($P < 0.05$) for the factors “Age” and “Group” and a combination of both by ANOVA (Table 1). Separate analysis of the age groups by *t*-test revealed that significant differences ($P < 0.025$) between feeding groups were only found at day 28 PI. Probiotic fed animals had a smaller proportion of IEL in the basal level and a higher proportion at the nuclear level (Table 1). Separate analysis of the feeding groups by *t*-test (not shown in Table 1) revealed that significant differences ($P < 0.025$) between age groups were only found in probiotic fed animals. At day 28 PI these animals had a smaller proportion of IEL at the basal level (villus tip $P = 0.057$; villus base $P = 0.008$) and a greater proportion at the nuclear level (villus tip $P = 0.076$; villus base $P = 0.004$) compared to the corresponding locations at DPI 2. In Supplemental Fig. 3, the abundance of IEL in villus tip and villus base is shown for absolute as well as relative numbers (influence of probiotic treatment – Supplemental Fig. 3A and B; influence of time post infection – Supplemental Fig. 3C and D).

Table 4.3-1: Table 1 - Morphological parameters and intraepithelial lymphocyte numbers: ►►►

The data is separated and grouped for the factors “day post infection” (DPI) and “feeding group” (control and probiotic). The respective significant differences are indicated and the corresponding illustration can be found in supplemental figures 2 and 3. The level of significance for ANOVA was α=0.05. As nearly all measurements were influenced by the time post infection, the age groups were additionally analysed separately via t-test and the level of significance was adjusted to 0.025 (Bonferroni adjustment). Intraepithelial lymphocyte (IEL) numbers are given in absolute numbers (IEL per 100µm basement membrane) as well as relative numbers (IEL in the respective intraepithelial compartment as % of the total IEL in this area).

Measured Parameters (and letter of corresponding boxplot)		2 DPI		28 DPI		Two-way ANOVA			t-test	
	Group	N	Mean	N	Mean	Age	Group	Age x Group	2 DPI	28 DPI
Villus length in µm (A)	Control	6	360.52	8	569.46	**.000***	.139	.349	.768	.037
	Probiotic	6	379.12	9	649.98					
Villus width in µm	Control	6	114.93	8	132.81	**.041***	.926	.873	.962	.852
	Probiotic	6	115.45	9	130.84					
Crypt depth in µm (B)	Control	6	211.88	8	272.37	**.001***	.680	.408	.458	.734
	Probiotic	6	227.73	9	267.04					
Crypt width in µm	Control	6	37.66	8	41.96	**.005***	.557	.406	.888	.223
	Probiotic	6	37.18	9	44.70					
Enlargement factor villi (C)	Control	6	4.71	8	6.53	**.000***	.099	.247	.688	.057
	Probiotic	6	4.97	9	7.92					
Enlargement factor crypts (D)	Control	6	5.23	8	6.22	.067	.728	.123	.328	.196
	Probiotic	6	5.58	9	5.67					
Ratio between enlargement factors (V:C) (E)	Control	6	0.91	8	1.08	**.003***	.125	.132	.981	.046
	Probiotic	6	0.91	9	1.41					
Basal IEL villus tip per 100µm (H)	Control	5	5.34	7	5.62	.114	.081	.289	.636	.058
	Probiotic	5	5.71	8	7.08					
Nuclear IEL villus tip per 100µm (H)	Control	5	2.07	7	2.37	**.005***	**.004***	**.029***	.363	**.004***
	Probiotic	5	2.39	8	4.40					
Apical IEL villus tip per 100µm (H)	Control	5	1.01	7	1.43	.096	.450	.815	.717	.479
	Probiotic	5	1.16	8	1.71					
Total IEL villus tip per 100µm (F)	Control	5	8.42	7	9.41	**.007***	**.011***	.090	.408	.007*
	Probiotic	5	9.26	8	13.19					
Basal IEL villus base per 100µm (I)	Control	4	5.97	7	6.55	.188	.559	.714	.918	.340
	Probiotic	4	6.10	8	7.12					
Nuclear IEL villus base per 100µm (I)	Control	4	1.93	7	1.97	**.023***	**.001***	**.027***	.247	**<.001***
	Probiotic	4	2.58	8	4.58					
Apical IEL villus base per 100µm (I)	Control	4	0.63	7	0.80	.187	**.008***	.499	.128	.014*
	Probiotic	4	1.19	8	1.71					
Total IEL villus base per 100µm (G)	Control	4	8.53	7	9.32	**.049***	**.016***	.198	.498	.003*
	Probiotic	4	9.87	8	13.40					
Basal IEL villus tip in %	Control	5	63,10	7	59,37	.060	.269	.527	.769	.189
	Probiotic	5	61,82	8	54,35					
Nuclear IEL villus tip in %	Control	5	24,60	7	25,40	**.041***	**.029***	.101	.732	.004*
	Probiotic	5	25,83	8	32,74					
Apical IEL villus tip in %	Control	5	12,29	7	15,23	.468	.773	.717	.965	.608
	Probiotic	5	12,34	8	12,91					
Basal IEL villus base in %	Control	4	70,15	7	70,79	.179	**<.001***	.114	.099	<.001*
	Probiotic	4	62,02	8	53,75					
Nuclear IEL villus base in %	Control	4	22,34	7	20,92	.143	**<.001***	**.032***	.207	**<.001***
	Probiotic	4	26,28	8	33,84					
Apical IEL villus base in %	Control	4	7,52	7	8,28	.704	**.011***	.934	.077	.058
	Probiotic	4	11,71	8	12,41					

* $p < 0.05$ for Two-way ANOVA and *$p < 0.025$ for t-test.

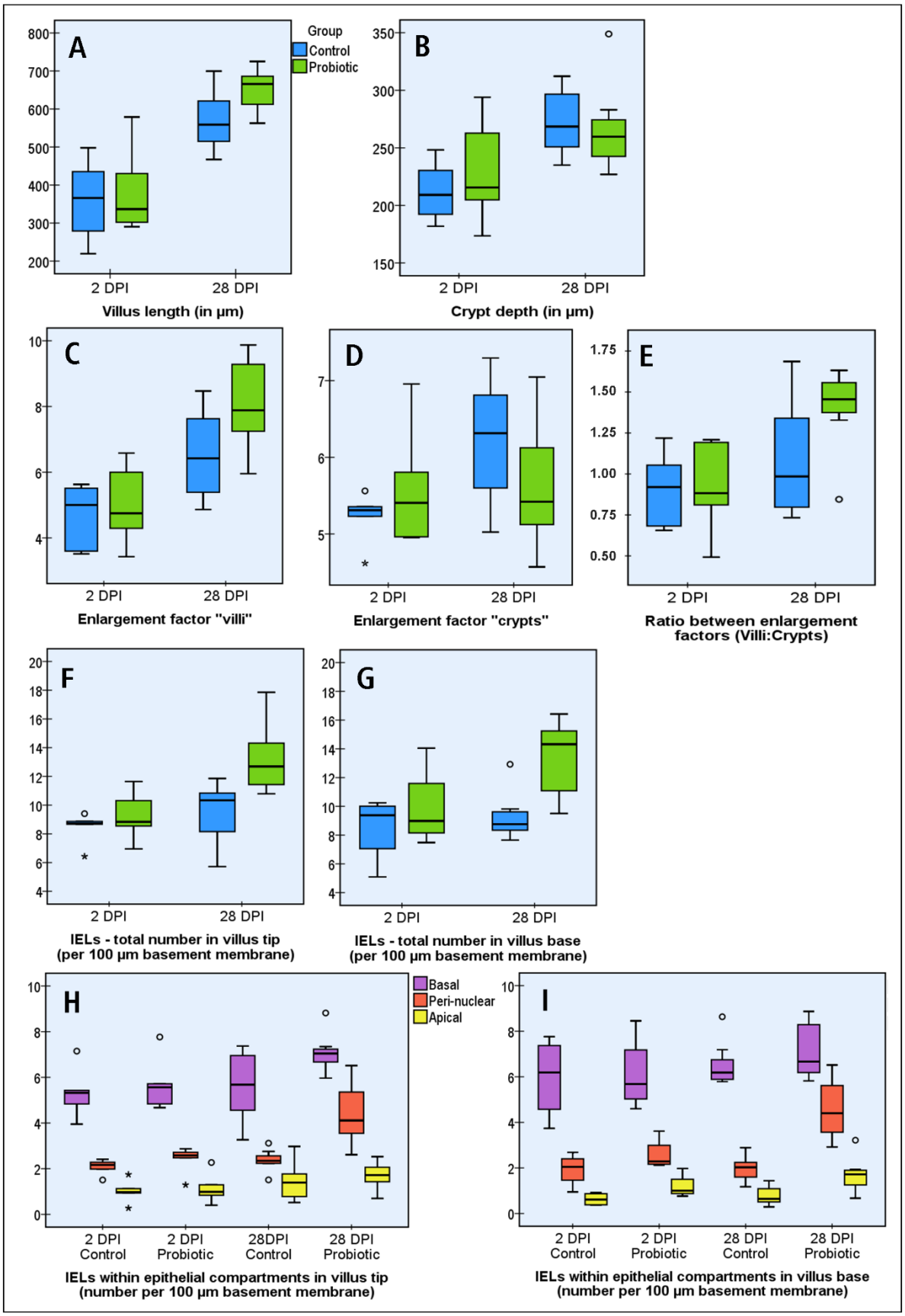


Figure 4.3-2: Supplemental figure 2 - Comparison of morphometric parameters:

A-E show the results for morphological parameters and F-I represent the data recorded for intraepithelial lymphocyte numbers separated and grouped for the factors “day post infection” (DPI) and “feeding group” (control and probiotic). The respective significant differences are indicated in table 1.

Evaluating the intravillus distribution of IEL, we compared measurements from villus tip and villus base via Paired Samples Test. Absolute numbers were found to be homogeneously distributed over all age and feeding groups, whereas relative numbers were not (Table 2). By analysing the test results for the influence of age, no differences could be detected. Also relative numbers in the probiotic feeding group showed no differences. However, in the villus tips of the control group, significantly ($P < 0.025$) less IEL at the basal level and more IEL at the nuclear and apical level were found compared to the villus base. By analysing the test results even further for the combined effects of age and feeding group, significant differences ($P < 0.025$) were only detectable in the 28 DPI control animals for the basal ($P = 0.148$ absolute, $P = 0.025$ relative) and nuclear ($P = 0.023$ absolute, $P = 0.025$ relative) IEL. See Supplemental Fig. 3 to visualise these descriptions.

Table 4.3-2: Table 2 - Paired Samples Test for intravillous distribution of IEL comparing measurements from villus tip and villus base:

Absolute numbers were homogeneously distributed over all age and feeding groups whereas relative numbers were not. Separating the test results for the influence of age (DPI =days post infection), no differences could be detected. Separating the test results for the influence of the feeding group (group), relative numbers in the probiotic feeding group were evenly distributed. In the control group, significantly fewer basal level IEL and more nuclear and apical level IEL were found in the villus tip compared with the villus base. Separating the test results even further (not shown in table 2) for the effects of age and feeding group together, significant differences (P< 0.025) were only detectable in the 28 DPI control animals for the basal (P=0.148 absolute, P=0.025 relative) and nuclear (P=0.023 absolute, P=0.025 relative) IEL.

Paired Samples Test		Basal IEL villus tip vs. base		Nuclear IEL villus tip vs. base		Apical IEL villus tip vs. base		Total IEL villus tip vs. base	
		absolute	relative	absolute	relative	absolute	relative	absolute	relative
Over-all (α=0.05)		.055	**.018***	.905	.332	.067	**.008***	.667	
Separated for age (α=0.025)	2 DPI	.066	.142	.647	.943	.234	.070	.552	
	28 DPI	.206	.065	.703	.243	.173	.056	.891	
Separated for group (α=0.025)	Control	.051	**.004***	.045	**.020***	.040	**.013***	.993	
	Probiotic	.587	.939	.486	.341	.769	.302	.592	

* p < 0.05 for over-all results of the Paired Samples Test and *p < 0.025 for the separated results of the Paired Samples Test.

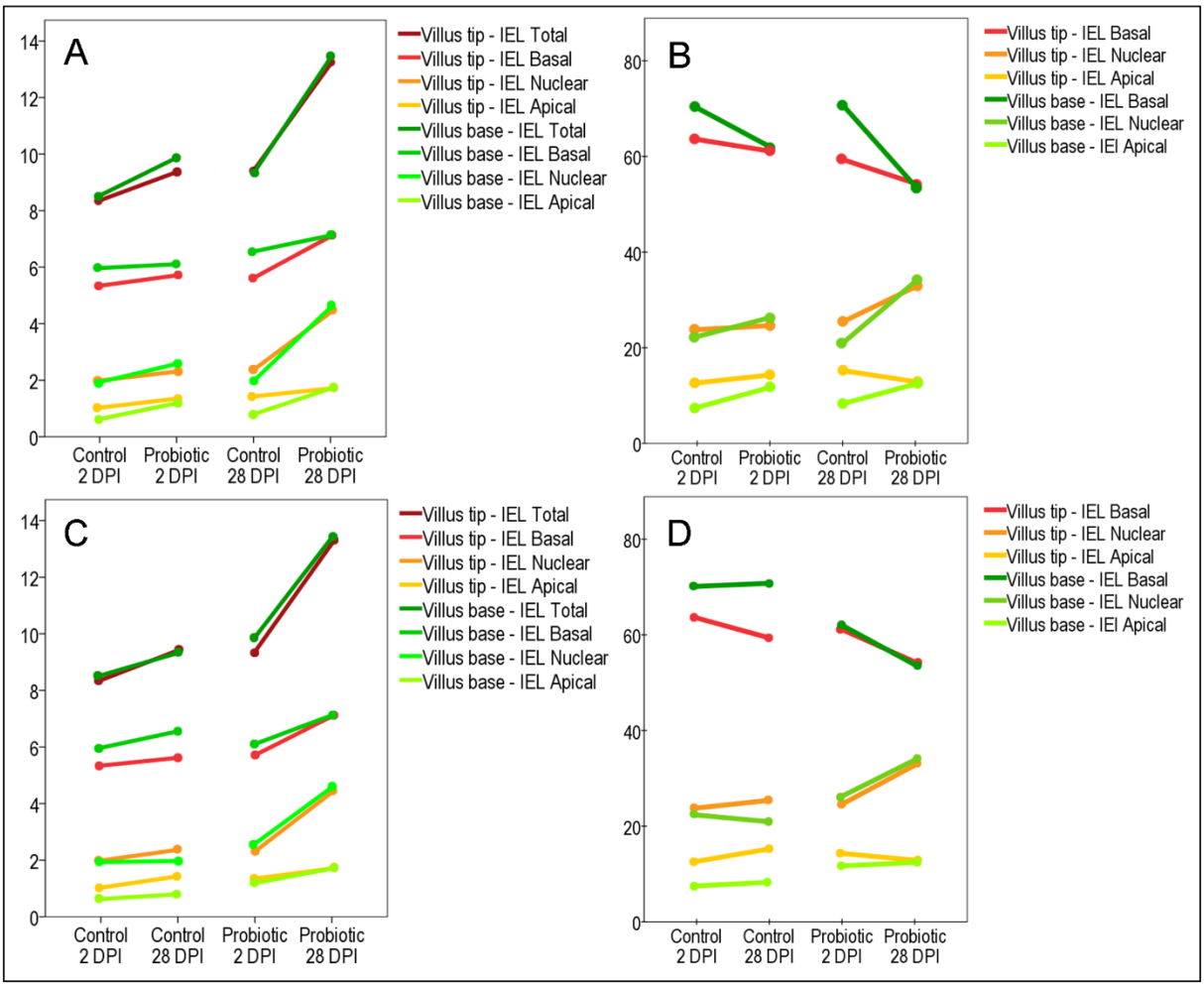


Figure 4.3-3: <u>Supplemental figure 3</u> - Mean values of IEL numbers for the different epithelial compartments within the epithelium in villus tip and villus base - comparison of absolute (A, C) and relative (B, D) numbers:

Influence of probiotic treatment - A, B; Influence of time post infection - C, D.

This simplified mean value plot graphically depicts the differences when using absolute and relative numbers. The respective significant differences are to be found in Table 1 and in the text.

Evaluation of absolute numbers (A) shows a higher number of IEL in the probiotic group in all assessed compartments in the villus tip as well as in the villus base. In comparison evaluation of relative numbers (B) shows a decrease of the basal IEL abundance in the probiotic group. A similar observation can be made for the change of IEL numbers within the feeding groups over time. The absolute numbers (C) show an increase in all compartments for 28 DPI while the relative numbers (D) in the basal IEL fraction decrease towards 28 DPI. While the mean values of the probiotic group are closely grouped, results from the control group are not. Assuming that a greater villus length and a higher villus / crypt ratio is the desired condition as well as that there is a functional connection to the resident IEL, the observed pattern of the probiotic group would be a desirable condition. In the probiotic group, the IEL were homogenously distributed along villus tip and base, while in the control group they were not. The IEL distribution in villus tips of control animals was more similar to the one in the probiotic animals (proportional reduction in basal IEL and increase in nuclear IEL over time) than to the one in the basal part of the villi.

DPI=days post infection

4.4. Discussion

Salmonella Typhimurium infection is usually a self-limiting disease in pigs. Nevertheless, this pathogen represents a threat due to its potential to persist hidden in the porcine body for long periods (Boyen et al., 2008). Formerly, auxinic antibiotics were used as feed additives to prevent infection outbreaks in pig production. Increasing rates of microbial resistances against such antibiotics have led to the ban on their usage in the European Union since 2006 (Anadón, 2006). Since then, much interest has been raised in the pursuit of potential alternatives such as probiotics (Castillo et al., 2012). Probiotics are believed to have a beneficial effect on the gastrointestinal tract, for example via modulation of the immune system and regulation of the surface epithelium's cellular growth and apoptosis (Kreuzer et al., 2012, Resta-Lenert and Barrett, 2003, Walsh et al., 2012 and Yan et al., 2007).

4.4.1. Pathological findings

Salmonella penetrates the intestinal epithelium preferentially through the Peyer's patches or solitary lymphoid tissue of the small intestine (Reis and Horn, 2010). As described in several earlier investigations (Coombes et al., 2005, Letellier et al., 2001, Santos et al., 2011 and Walsh et al., 2012), and in our study, *Salmonella* caused damage of the intestinal mucosa at the preferred entry sites. Pathological alterations were particularly severe at day 2 PI, especially in the ileum and colon where the most characteristic inflammatory lesions were found. In the older animals, the intestinal mucosa appeared to have largely recovered towards its normal morphological and physiological status. However, it remains unclear whether the time the intestine needed to recover was the same in both groups, as no samples were taken between 2 and 28 DPI. The changes in the tonsilla veli palatini suggest that despite recovery of the intestine, immune reactions were still in progress at day 28 PI.

4.4.2. Morphometric parameters were influenced by the time post infection (age) and probiotic feeding

The proximal small intestine is the primary site of digestion and absorption in the small bowel (Caspary, 1992 and Sawaya et al., 2012). The size of the small intestinal surface area is commonly thought to be related to its functional capacity and has to be considered in nutritional studies (Mayhew and Middleton, 1985 and Stenling and Helander, 1981). Villus and crypt related morphological parameters give an indication of the maturity and functional capacity of the enterocytes (Tang et al., 1999). An increase in the ratio between villus length and crypt depth corresponds to an increase in digestion and absorption (Gu et al., 2002 and Navidshad et al., 2010). We suggest that the same principle holds true for the ratio between villus and crypt enlargement factors.

Nearly all measured morphometric parameters were influenced significantly by the time post infection. After day 28 PI, quantitative measurements revealed longer and wider villi, deeper and wider crypts, a higher villus enlargement factor and a higher ratio between villus and crypt enlargement factors compared to measurements after day 2 PI. We also compared our measurements of jejunal villus length at day 2 PI (≈370 µm 40 d/12 dpw (40 day old pigs 12 days post weaning)) with observations from our earlier trials without a *Salmonella* challenge (≈374 µm 34–40 d/8–12 dpw (Liu et al., 2014, Martin et al., 2012 and Reiter, 2006)) and literature sources (≈420 µm 32–42 d/12–26 dpw (Arnal et al., 2014, Levesque et al., 2012, Nofrarías et al., 2006 and Pluske et al., 1997)). The comparison shows that jejunal villus length at day 2 PI can be judged to be in a normal range or slightly shortened. Jejunal crypt depth and crypt enlargement factor measured at day 2 and 28 PI were in a normal range compared to our trials without *Salmonella* challenge (Liu et al., 2014, Martin et al., 2012 and Reiter, 2006). Likewise, we compared our measurements of jejunal villus length from day 28 PI (≈570 µm control/≈650 µm probiotic 66 d/38 dpw) with observations from our earlier trials without *Salmonella* challenge (≈513 µm 54–56 d/26 dpw (Liu et al., 2014, Martin et al., 2012 and Reiter, 2006)) and literature sources (≈527 µm 40–150 d/28–95 dpw (Agyekum et al., 2012, Arnal et al., 2014, Hedemann et al., 2005, Letellier et al., 2001, Levesque et al., 2012, Price et al., 2010 and Suo et al., 2012)). The comparison at day 28 PI shows that jejunal villus length, especially that of the probiotic treatment group, can be judged to be lengthened to some extent. Also the villus enlargement factor measured at day 28 PI, especially that of the probiotic feeding group (7.92 66 d/38 dpw), was somewhat bigger than that (≈6.08 56 d/26 dpw) in observations from an earlier trial without *Salmonella* challenge (Reiter, 2006). The comparison with results from non-challenge trials showed that jejunal morphology was mainly normal and that the biggest changes can be attributed to an age dependant development. Probiotic treatment showed minor effects after DPI 28 in form of tendentially longer villi and a higher ratio of villus enlargement factor/crypt enlargement factor. As an increased rate of epithelial renewal is an important defensive mechanism in response to invading pathogens that damage epithelial cells (Tang et al., 1999), this could be interpreted as an advantage. Nonetheless, activation and mobilisation of immune cells to migrate actively towards or into the intestinal lumen requires an increased energy supply for these cells (Dugan et al., 1994 and MacIver et al., 2013). This increased demand could have resulted in expanded proliferation of the epithelium.

4.4.3. IEL quantities and distribution were altered by time post infection and probiotic feeding

IEL are potentially the first immune cells to encounter *Salmonella* during infection via the oral route (Davies et al., 2004). They are responsible for maintaining the intestinal epithelial surface integrity and homeostasis (Mattapallil et al., 1998, van Wijk and Cheroutre, 2009 and Yu et al., 2012).

The immune system continuously changes as the young pig matures. Quantitative and phenotypical alterations of immune cell populations, including increases in IEL numbers during development, have been described (Brown et al., 2006, Rothkötter et al., 1999 and van Wijk and Cheroutre, 2009). IEL numbers have also been demonstrated to increase after oral infection with *S. enterica* serovar Typhimurium (Li et al., 2012). Thus, the increase of IEL in older animals observed in our study reflects both a maturation and an infection-driven process.

Vega-López et al. (2001) have studied the localisation and development of IEL in pigs. They have found over half of these IEL (52% duodenum, 53% ileum) localised at the epithelium's basement membrane, numerous (43% duodenum, 42% ileum) at the enterocyte nuclear level and relatively few (5%) apically in the epithelium. These proportions were homogeneously maintained along the villi's tip, middle and bottom sections (Vega-López et al., 2001). Similar proportions have been found in our trial examining the jejunum, although a slightly different numerical distribution pattern could be observed. This might be a consequence of the challenge situation, the different age of the piglets as well as the different localisation within the small intestine. Whether the differences between probiotic and control group concerning the distribution of IEL along the villi found in our study are of biological relevance is a matter of speculation. One may conclude that the villus tip with the more developed enterocytes has nearly reached an optimal functional and morphological state and that the villus base with the younger enterocytes is still in a process of regenerative change. This process seems to have happened faster and more effectively in the probiotic group.

In agreement with our results, several studies have reported an increase in number of lymphocyte populations, especially IEL, after probiotic treatments (Babinska et al., 2005, Bai et al., 2013, Dalloul et al., 2003, Deng et al., 2012, Lee et al., 2010, Levkut et al., 2012, Pirarat et al., 2011, Roselli et al., 2009 and Scharek et al., 2007). However, there are also reports on a reduction of intestinal IEL after feeding probiotics (Ashraf et al., 2013, Lee et al., 2010 and Mafamane et al., 2011).

Interestingly, Mafamane et al. (2011) using flow cytometry found a reduced number of jejunal CD8αβ+ IEL in piglets treated with *E. faecium* in a similar *S*.Typhimurium challenged study. This does not necessarily contradict our results: $CD8^+$/$CD8\alpha\beta^+$ IEL have been found to be located at the basement membrane within the epithelial compartment (Helgeland et al., 1997 and Vega-López et al., 2001). Since absolute numbers of IEL in this compartment were

not reduced in our experiments, one may conclude that the CD8αβ$^+$ IEL population was unaffected in our study. However, when relative numbers were taken into consideration, a significantly smaller percentage of IEL in the basal intraepithelial compartment was detected at 28 DPI in the probiotic feeding group. Since flow cytometry results are usually given in relative numbers, our results are consequently in agreement with those of Mafamane et al. (2011). Additionally, Mafamane et al. have suggested a reduction in the absolute numbers of CD8αβ$^+$ cells. However, since histology is considered to be the most reliable way to count individual IEL (Peaudecerf and Rocha, 2011), this mainly demonstrates how difficult it is to compare absolute and relative numbers as well as different experimental setups and methods. It also raises the question whether flow cytometry of isolated cells alone can depict processes in complex tissues like the intestine.

Considering the prominent significant difference in IEL numbers at the nuclear level between probiotic and control groups, we assume that this population might be of great functional importance in piglets. This population was found to be CD4$^-$CD8$^-$ in pigs and CD3$^-$CD8αα$^+$ in rats (Helgeland et al., 1997 and Vega-López et al., 2001). Using a simplified classification of IEL based on phenotype and gene expression (Hayday et al., 2001), the basal CD8αβ$^+$ IEL belong to the “conventional type”, i.e., antigen experienced cells originating from peripheral T cells and homing to the gut mucosa. They have immunologic memory function and mount an adaptive response. The IEL found at the nuclear level belong to the “unconventional type”. They have functions in-between adaptive and innate responses, with one being most interesting in the context of our study, namely the protection of epithelial integrity (Hayday et al., 2001 and Peaudecerf and Rocha, 2011). Edelblum et al. (2012) demonstrated the ability of an IEL population fitting to the “unconventional type” to contact multiple epithelial cells over a short time and thus provide a potential mechanism by which they could prevent epithelial injury and infection (Edelblum et al., 2012). For non-challenged piglets, our research group already described that *E. faecium* enhanced intestinal barrier function (Klingspor et al., 2013). And since IEL profoundly regulate induction of epithelial apoptosis or growth (Swamy et al., 2010), we suggest that the nuclear IEL in the probiotic fed group possibly had a positive influence on epithelial integrity resulting in improved morphological features at 28 DPI.

4.5. Conclusion

In the present study we observed that in *Salmonella* challenged piglets the supplementary feeding of *Enterococcus faecium* NCIMB 10415 resulted in a significantly increased number of intraepithelial lymphocytes. The most prominent differences in IEL numbers were found in the population of IEL located at the enterocytes nuclear level. This IEL population may be a

special subpopulation involved in the maintenance of epithelial integrity. The results indicate an immunomodulatory effect of *E. faecium*. The slight increase of jejunal villus length and villus/crypt enlargement factor ratio in probiotic fed animals may be the effect of a more efficient mucosal regeneration after the infection but no beneficial effect could be observed in the early infection phase. Additionally, we conclude that in future studies it may be necessary to combine the efficient phenotypical characterisation of cell populations via flow cytometry with the in situ characterisation and quantification via histology.

Author contributions

J. Rieger: conception and design of the study; critical literature review; acquisition of data; analysis and interpretation of data; draft of manuscript; final approval

P. Janczyk: conception and design of the study; acquisition of data; draft of manuscript; final approval

H. Huenigen: conception and design of the study; analysis and interpretation of data; critical review; final approval

K. Neumann: analysis and interpretation of data; critical review; final approval

J. Plendl: conception and design of the study; analysis and interpretation of data; draft of manuscript; final approval

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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5. Enhancement of immunohistochemical detection of *Salmonella* in tissues of experimentally infected pigs

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Enhancement of Immunohistochemical Detection of *Salmonella* in Tissues of Experimentally Infected Pigs

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Abstract

Salmonella Typhimurium is one of the main pathogens compromising porcine and human health as well as food safety, because it is a prevailing source of foodborne infections due to contaminated pork. A prominent problem in the management of this bacteriosis is the number of subclinically infected carrier pigs. As very little is known concerning the mechanisms allowing *Salmonella* to persist in pigs, the objective of this study was to develop an immunohistochemical approach for the detection of salmonellae in tissue of pigs

experimentally infected with *Salmonella* Typhimurium. Samples were obtained from a challenge trial in which piglets of the German Landrace were intragastrically infected with *Salmonella enterica* serovar Typhimurium DT104 (1.4-2.1×10^{10} CFU). Piglets were sacrificed on days 2 and 28 post infection. Tissue samples of jejunum, ileum, colon, ileocecal mesenteric lymph nodes (*Lnn. ileocolici*), and tonsils (*Tonsilla veli palatini*) were fixed in Zamboni's fixative and paraffin-embedded. Different immunohistochemical staining protocols were evaluated. *Salmonella* was detected in varying amounts in the tissues. Brown iron-containing pigments in the lymph nodes interfered with the identification of *Salmonella* if DAB was used as a staining reagent. Detergents like Triton X-100 or Saponin enhanced the sensitivity. It seems advisable not to use a detection system with brown staining for bacteria in an experimental setup involving intestinal damage including haemorrhage. The use of detergents appears to result in a higher sensitivity in the immunohistochemical detection of salmonellae.

Keyword

Swine, immunohistochemistry, histochemistry, bacteria, detection limit

5.1. Introduction

Salmonella is an important pathogen threatening porcine and human health as well as food safety. Amid the most pervasive sources of foodborne diseases, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is especially strongly linked with human disease caused by the consumption of contaminated pork.[1-3] In pigs, an *S.* Typhimurium infection triggers clinical symptoms with enterocolitis, often followed or accompanied by sub-clinical infections of *silent carrier* animals that can function as a reservoir, infect other animals and transmit the pathogen to the food chain.[4-6] *Salmonella* infects cells lining the epithelial layer of the small and large intestine such as M-cells, absorptive enterocytes or goblet cells and may cross this barrier *via* different mechanisms to invade the *lamina propria*.[7-9] After reaching the *lamina propria* of the intestinal mucosa, *Salmonella* is mainly taken up by macrophages in which they then replicate in a protected intracellular niche and which may also transmit the bacteria to other organs.[10,11] Salmonellae harbour a sophisticated arsenal of mechanisms to survive and replicate in the host. Although bacterial persistence is a key phase of a pathogen's life cycle and represents an opportunity for disease control, very little is known about how the pathogen survives for long periods of time in the mammalian host in the presence of immunosurveillance.[12] In order to study the largely unknown mechanisms used by *Salmonella* to persist in pigs[5] and particularly to trace *Salmonella's* route through the body, it is a great challenge to reliably mark and track salmonellae in histological sections of different organs and tissues. As part of a big research consortium, one aim of our working group was the demonstration of *S.* Typhimurium in paraffin embedded tissues from experimentally infected pigs. Since immunohistochemistry represents a suitable approach to do this,[13] we evaluated several protocols for applicability which yielded very heterogeneous results. The refined immunohistochemical protocol is presented. Further information concerning other aspects of the same experiment may be found elsewhere.[14,15]

5.2. Materials and Methods

5.2.1. Sample collection

The samples for this study were obtained during a *Salmonella* challenge trial already described.[14] In short, samples were obtained from a probiotic feeding trial in which piglets of the German Landrace were intragastrically challenged with *Salmonella enterica* serovar Typhimurium DT104 (1.4-2.1×10^{10} CFU). Piglets from each group were sacrificed on days 2 and 28 post infection (DPI). The animals were euthanized by an overdose of pentobarbiturates (Narcoren, Merial GmbH, Germany) under general azaperone (Stresnil, Janssen Animal Health, Neuss, Germany) -ketamine (10% ketamine, Bremer Pharma GmbH, Warburg,

Germany) anaesthesia. Samples of mid-jejunum, ileum, colon ascendens, ileocecal mesenteric lymph nodes (*Lnn. ileocolici*), and tonsils (*Tonsilla veli palatini*) were taken within 15 min after sacrifice and treated as already described.[14,16] All samples were rinsed in ice-cooled Ringer solution. Intestinal samples were cut open on the mesenterial side, trimmed to squares and pinned on cork pieces with the mucosal side facing upwards. The tissues were fixed for 26h in Zamboni's fixation solution and rinsed in PBS, dehydrated in a graded series of ethanol, embedded in paraffin, cut to 5 µm thin sections, mounted on HistoBond® slides (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), dewaxed in xylene and rehydrated in a decreasing series of ethanol. Experimental approval had been given by the local authority / Regional Office for Health and Social Affairs Berlin (Landesamt für Gesundheit und Soziales, Berlin ID: G0348/09).

5.2.2. Immunohistochemistry

Different immunohistochemical staining protocols with a monoclonal mouse anti-*Salmonella* Typhimurium antibody (Mouse-anti-Salmonella Typhimurium, monoclonal, Maus-IgG, 5mg/mL, Clone 8C11C, Acris Antibodies GmbH, Herford, Germany) were evaluated. Two protocols are presented here for comparison (Table 1).

5.2.3. Pigment differentiation / iron demonstration

For the identification of the gold-brown pigments encountered in lymph nodes, the very same histological samples employed for immunohistochemistry were used. For the demonstration of iron, Berlin blue method (trivalent iron) and Turnbull blue method modified according to Quincke (bi- and trivalent iron) were applied.[17] Lipofuscin was detected according to Hueck and Pearse.[18] To validate the staining protocols, liver samples of goat, sheep, cow or rat, available in our institute from stock, were used as positive controls. The liver was chosen, since a number of different pigments may be seen as an incidental finding within hepatocytes and Kupffer cells, amongst them lipofuscin and hemosiderin.[19]

Table 5.2-1: Table 1 - Comparison of immunohistochemical protocols:

If not stated otherwise, all steps were carried out at room temperature. All incubation steps were carried out in a humid chamber. As additional positive control, cultured bacteria were used; therefore *Salmonella* containing culture medium was dropped on slides and heat fixed. The slides where treated in the same way as the tissue sections.

Protocol 1	**Protocol 2**
Rinsing of the rehydrated sections in 0.01M citrate buffer, pH 6.0	
Heat induced epitope retrieval: 0.01M citrate buffer, pH 6.0, 96°C, 25'	
Cooling down of the section container 15' at room temperature and 5' in cold tap water	
Rinsing of the sections in Aq. Bidest 2'	
Surrounding of sections with a hydrophobic barrier using a barrier pen (S2002, Dako Deutschland GmbH, Hamburg, Germany)	
Rinsing of the sections in TBS (0.05M Tris-HCl pH 7.6 + 0.9% NaCl) 2x 3'	
Peroxidase-block: 3% H_2O_2 in TBS 20'	
Rinsing in TBS 5'	
Rinsing in TBS + 0.05% Tween20® 5'	Permeabilization: PBS + 0.1 % saponin **or** PBS + 0.1% Triton X-100 20'
Preincubation: 3% BSA + 5% normal goat serum inTBS 30'	Preincubation: 3% BSA in PBS 20'
Primary antibody incubation: 1:2500 in TBS + 1% BSA + 2% normal goat serum Isotype control (mouse IgG1, DAKO X0931, 100µg/ml, Dako Deutschland GmbH, Hamburg, Germany): 1:50 in TBS + 1% BSA + 2% normal goat serum Buffer control: TBS + 1% BSA + 2% normal goat serum overnight at 4°C	Primary antibody incubation: 1:5000 in PBS + 1% BSA Isotype control (mouse IgG1, DAKO X0931, 100µg/ml, Dako Deutschland GmbH, Hamburg, Germany): 1:500 in PBS + 1% BSA Buffer control: 1% BSA in PBS overnight at 4°C
Rinsing in TBS 1x 3'	Rinsing in PBS 1x 3'
DAKO EnVision+-System/HRP labelled goat-anti-mouse-polymer (DAKO K4001, Dako Deutschland GmbH, Hamburg, Germany) was applied for 40min at RT in the dark.	
Rinsing in TBS 1x 5' and PBS 1x 5'	Rinsing in PBS 2x 5'
HRP detection: DAB 30' in the dark **or** Histogreen (Linaris, Wertheim-Bettingen, Germany) 10'	HRP detection: Histogreen (Linaris, Wertheim-Bettingen, Germany) 10'
Rinsing in PBS 2x3'; Rinsing in Aq. Dest	
Counterstaining: Haemalaun AD Mayer for ~12" in Aq. Bidest, 2' bluing in tap water	
Dewater: 100% ethanol I, II, III each 60", Xylene I 2', Xylene II 1'	
Addition of mounting medium and coverslip	

5.3. Results

5.3.1. Immunohistochemical detection of *Salmonella*

S. Typhimurium was detected immunohistochemically in varying amounts depending on the time post-infection, tissue localization and the protocol used. The positively stained objects appeared as roundish to longish particles with a diameter of ~2 µm. With protocol 1, single bacteria were observed lying freely between cells of tissues as well as intracellularly, often in cells with the morphology of macrophages (Figure 1). Salmonellae were abundant in the tunica mucosa of ileum and colon 2 DPI, where several bacteria appeared to group in clusters. The ileal domes were particularly frequented by the pathogen. *S.* Typhimurium was found in lymph nodes, albeit in low numbers, and was not detectable in tonsils. In addition to the immunohistochemically labelled salmonellae, spots of brown pigment with the same size as the bacteria were visible in sections of lymph nodes. They also appeared in the control sections (Figure 2C). As the brown iron-containing pigments (see below) in the lymph nodes interfered with the identification of *Salmonella* if DAB was used as a staining reagent, HistoGreen was used instead, which labels the targeted bacteria in a bright green-blue color (Figure 2 D,E).

After implementing a permeabilization step with detergents like Triton X-100 or Saponin (protocol 2), we found that the sensitivity was considerably enhanced. More staining signals were visible and we also found the bright green-blue color easier to recognize (Figure 3). In addition to a higher amount of *Salmonella*, which could now be detected in ileal and colonic tissues, it was now frequently possible to show the presence of bacteria in lymph nodes and tonsils. In lymph nodes and tonsils the staining signals were dispersed throughout the tissue and the bacteria appeared not to be grouped in clusters (Figure 3D). In the positive controls derived from cultured *S.* Typhimurium, the staining intensity between single bacteria varied notably. Whereas approximately 1% of the bacteria exhibited a strong positive reaction and circa 5-10% exhibited a moderate staining signal, most cells showed very weak or almost invisible staining grades. The strongly stained bacteria were the biggest ones. They were rodshaped and the staining signal was situated at the perimeter of the cells. The moderately stained cells appeared to be a little smaller and were also rod shaped. Bacteria with weak or nearly no staining appeared to be the smallest ones and exhibited a more roundish shape. A similar phenomenon could be observed in the tissue samples, in which the staining intensity differed between single bacteria in the gut lumen and inside the tissue (Figure 3C).

5.3.2. Pigment differentiation / iron demonstration

In the above mentioned samples of lymph nodes, particularly in their medulla, spots of brown pigment were visible, which had the same size as the expected immunohistochemical reaction product for *S.* Typhimurium (Figure 2 A,C). Iron deposits were positively demonstrated using Turnbull blue and Berlin blue (Figure 2B). Lipofuscin reaction was negative. Positive staining reactions for lipofuscin as well as iron were detectable in the positive controls.

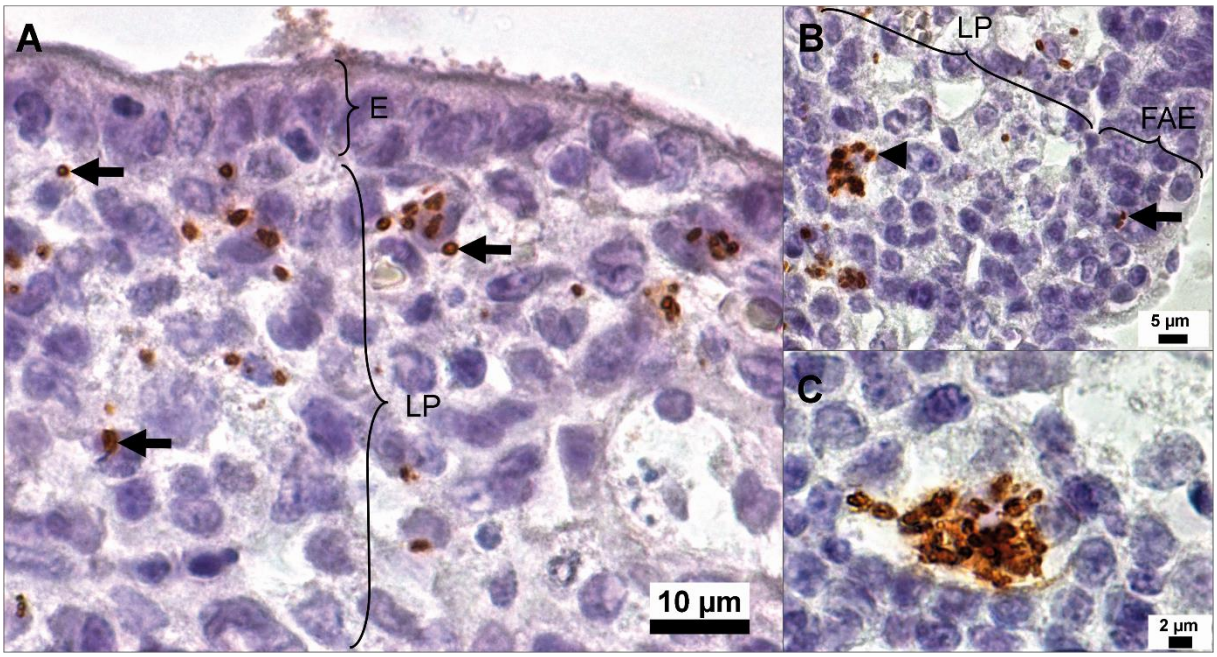

Figure 5.3-1: Figure 1 – Immunohistochemistry:

A) *Salmonella* (arrows) can be seen as ca. 2 µm big objects outlined in brown staining with DAB in the ileal mucosa directly under the epithelium (E) in the *lamina propria* (LP).

B) Ileal dome, with follicle associated epithelium (FAE). Bacteria are situated within the FAE (arrow) and in the underlying *lamina propria* (LP) (arrowhead).

C) A *Salmonella* containing cell, morphologically resembling a macrophage, in the *lamina propria* of the ileum is illustrated.

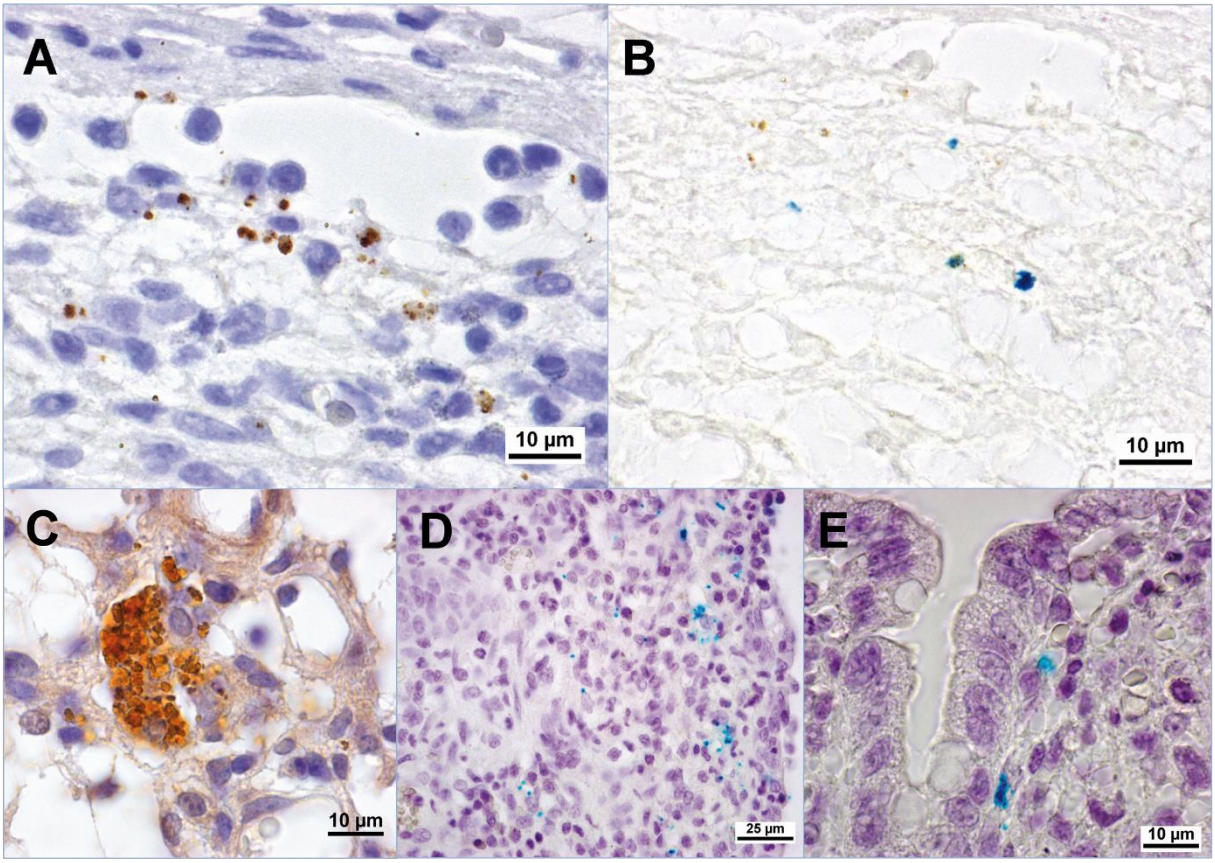

Figure 5.3-2: **Figure 2 - Pigment differentiation:**

A, B) Subcapsular region of a lymph node.

A) Numerous brown staining signals can be observed after staining with protocol 1 but similar ones were also present in IgG as well as buffer control samples (-> C).

B) Neighbouring section next to A. First immunohistochemistry followed by iron demonstration with Berlin blue was carried out on the same slide. Blue reaction product identifying iron and brown staining signal of immunohistochemistry can be distinguished.

C) Gold-brown staining signals in the medullary region of a lymph node of a control section. The brown pigments could be observed in such concentrated form and as single objects disseminated throughout the tissue. Please also compare this picture to figure 1C.

D, E) Ileal tissue, in which *Salmonella* was demonstrated with a green-blue (HistoGreen) instead of a brown reaction product.

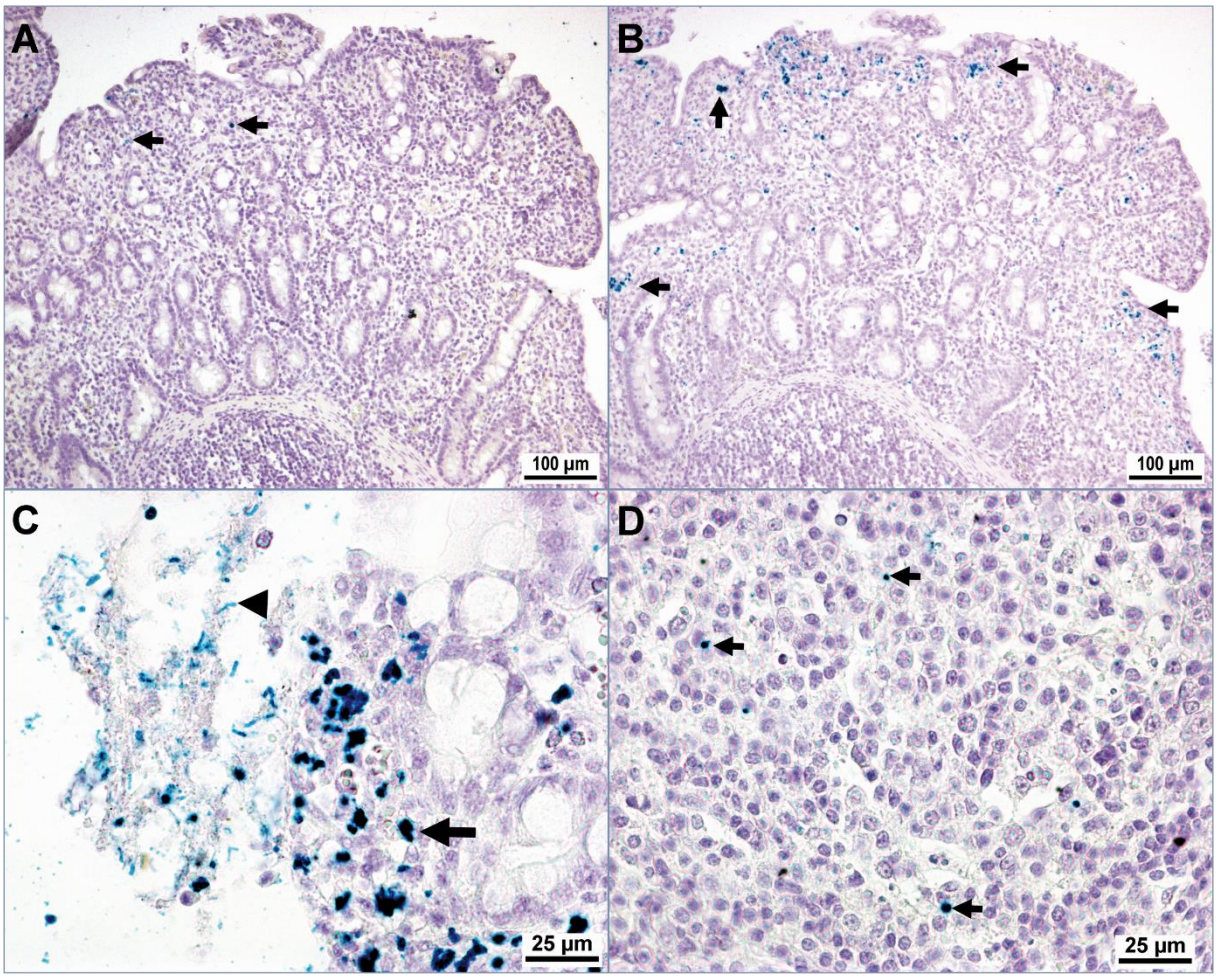

Figure 5.3-3: **Figure 3 - Influence of detergents on the amount of reaction product:**

(*Salmonella* = green-blue staining signals).

A) Ileal mucosa not treated with detergent. Few salmonellae are visible (arrow).

B) Serial section next to A. Trition X-100 was used in the staining protocol as a detergent. Numerous salmonellae are visible (arrow).

C) Higher magnification of ileal epithelium (B). Salmonellae are also visible in the intestinal lumen. Note the differences in staining intensity of single bacteria inside (arrow) and outside (arrowhead) of the tissue.

D) *Salmonella* (arrow) could also be demonstrated in the tonsils after introducing a detergent in the protocol. Without it, salmonellae were not detectable in this tissue.

5.4. Discussion

Histochemistry, especially immunohistochemistry, is a suitable approach to investigate the exact localization of a pathogen *in situ*. It enables the researcher to correlate its occurrence to e.g. pathologic lesions or other pathogens.[20,21] In the present study, *S.* Typhimurium was detected immunohistochemically in different porcine tissues of the intestine and in the tonsils. Frequently, the pathogen was spotted within cells, often in those which morphologically resembled macrophages. The staining results concerning bacterial morphology and distribution were principally comparable to descriptions found in the literature.[6,8,22-24] In the sections of lymph nodes including control sections (IgG - and buffer control), spots of brown pigment of approximately the same size as the expected immunohistochemical reaction product for *S.* Typhimurium were visible. Since these pigment granules were of similar size and color as the labelled bacteria, it was necessary to distinguish them from the microorganisms. The pigment granules were found to contain iron, presumably representing hemosiderin, which can be a result of mucosal haemorrhages.[25,26] Consequently, the detection system was changed to a green color to solve this problem, although it has to be noted that DAB gives a more crisp staining result compared to HistoGreen. It seems advisable not to use a detection system with brown staining in experimental setups involving intestinal damage including haemorrhage.

Immunohistochemistry is a powerful tool to demonstrate microorganisms in tissue samples;[27] however, an important question can be raised concerning its detection limit. As microorganisms are at the limit of light microscopical detection and results may vary from slide to slide because of heterogeneous distribution of the bacteria in the tissue, histology is not the method of choice for routine diagnosis and quantification of bacterial infections in tissues.[22] One method routinely used to detect and quantify microbes is microbial plate counting. Interestingly, an organ-specific difference between results of our immunohistochemical labelling of *S.* Typhimurium and microbial plate counting done in the same trial by Kreuzer *et al.* could be found.[2] 2 DPI levels around 10^3 CFU/g tissue could be quantified via plate counting in the tonsils, jejunum and lymph nodes, whereas *S.* Typhimurium was hardly detectable in these organs via immunohistochemistry using protocol 1. In contrast, immunohistochemistry of *S.* Typhimurium in ileal and colonic tissue 2 DPI was reliably possible. Plate counting done for these organs resulted in higher levels, namely between 10^4 - 10^6 CFU/g tissue. We therefore conclude that the number of CFUs was under the immunohistochemical detection limit in tonsils, jejunum and lymph nodes with protocol 1. In protocol 1 we applied only a low concentration (0.05%) of a mild membrane solubiliser (Tween 20) for a short time (5 min). In protocol 2 we applied a higher concentration (0.1%) of a mild detergent (Saponin) or a harsh detergent (Triton X-100) for a longer time (20 min). This modification enhanced the immunohistochemical sensitivity enormously and *S.* Typhimurium was also detectable in the

previously negative organs. The observation of sensitivity enhancement due to detergents stands in contrast to the statement that antigen retrieval is in general not required for the demonstration of bacteria in fixed tissues.[28] For example Searle, *et al.* used a permeabilization step for the immunocytochemistry but not for the immunohistochemistry to detect *Salmonella.*[29] In support of our findings, other studies also used detergents for histologic *Salmonella* demonstration, although for different applications, *e.g.*, cryosections, thicker sections or immunocytochemistry.[30-34] The rationale behind the use of detergents in immunocytochemistry and applications using thicker tissue sections or cryosections is to allow the antibody to reach the antigen if it is situated in a cell compartment shielded by a membrane, especially after aldehyde fixation.[35] Detergents are surface-active molecules that self-associate and bind to hydrophobic surfaces in a concentration-dependent mode.[36] For example for Saponin, it was demonstrated that through interaction with plasma membrane cholesterol, it makes cells permeable without major disruption of organelles, by literally opening pores in the plasma membrane when used in higher concentrations.[37] Within mammalian cells, *Salmonella* inhabits a membrane-bound vacuole known as the Salmonella-containing vacuole but also colonizes the cytosol of cells.[38] Therefore, we assume that a permeabilization step in the immunohistochemical protocol is necessary to access the bacteria situated in the cytosol as well as the ones in the membrane-surrounded vacuoles. Negative immunohistochemical results may otherwise be false negatives or the amount of detected bacteria artificially low.

To the best of our knowledge, there are very few published detection limits for the immunohistochemical identification of bacteria in histological samples (10^2 CFU g^{-1} tissue for mycobacteria in fish[39]). Based on our observations, we propose a detection limit of roughly 10^2-10^3 CFU per g tissue in our experimental setup. The detection limit may of course be different for *e.g.*, different antigens, targeted bacteria and chosen staining protocols, as was also demonstrated in this study. Since some recent studies used enzymes in their staining protocols, this could be another option to enhance sensitivity.[40-42] An additional reason for a varying immunohistochemical detection limit may be a potential change of surface structure of *S.* Typhimurium in different environments. The antibody used in this study was directed against heat-inactivated LPS from *S.* Typhimurium. As described in the Results section, staining signals from *S.* Typhimurium recovered directly out of the culture medium were heterogeneous and mostly weak, whereas those of tissue sections of the ileum and colon were strongly visible. We also noticed differences between the tissue resident bacteria and the ones in the intestinal lumen. The observed size differences between the variably stained bacteria could be attributed to the *Quellung* reaction.[22] Quellung (German word for *swelling*) is the result of the combination of the polysaccharidal bacterial capsule antigens with the specific antibody, resulting in an apparent capsule swelling.[43] For the fungus *Cryptococcus neoformans*, which is used as a system to study capsule reactions because it has a large polysaccharide capsule that is readily

visible by light microscopy, it was shown that distinct capsular reactions depend on the antibody epitope specificity and the yeast serotype.[44] Therefore different degrees of Quellung-reaction and resulting different detection sensitivities could also be possible in *Salmonella*-immunohistochemistry. It has been established that phase and antigenic variation lead to substantially altered heterogenic phenotypes of a clonal bacterial population. It has been shown that surface antigens in particular vary under differing conditions, even during the journey through the body, to avoid adverse immune reactions and establish long term persistence.[45] Another reason for the stronger staining signals detected inside of the intestinal tissues might be the tendency of *Salmonella* to form microcolonies,[46] thereby probably amplifying the antigen concentration in one spot. Additionally, the accumulation of dense material surrounding intracellular *S.* Typhimurium, supposedly originating from lysed bacterial products, was described in an transmission electron microscopical study.[47] This material could also amplify the staining signal.

In conclusion, the use of detergents seems to be necessary for the proper immunohistochemical detection of *Salmonella* in paraffin embedded tissues and enhances the identification sensitivity. Additionally it is advisable not to use a detection system with brown staining for bacteria in an experimental setup involving intestinal damage including haemorrhage.

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6. Porcine intestinal mast cells. Evaluation of different fixatives for histochemical staining techniques considering tissue shrinkage

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Porcine Intestinal Mast Cells. Evaluation of Different Fixatives for Histochemical Staining Techniques Considering Tissue Shrinkage

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Contributions

JR, design, critical literature review, data collection, analysis and interpretation, draft of manuscript, final approval

ST, design, data collection, statistical data analysis and interpretation, final approval;

HH, design, data analysis and interpretation, critical review, final approval

RMH, critical literature review, data interpretation, draft of manuscript, critical review, final approval

JP, design, data analysis and interpretation, draft of manuscript, critical review, final approval

Conflict of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Abstract

Staining of mast cells (MCs), including porcine ones, is critically dependent upon the fixation and staining technique. In the pig, mucosal and submucosal MCs do not stain or stain only faintly after formalin fixation. Some fixation methods are particularly recommended for MC staining, for example the fixation with Carnoy or lead salts. Zinc salt fixation (ZSF) has been reported to work excellently for the preservation of fixation-sensitive antigens. The aim of this

study was to establish a reliable histological method for counting of MCs in the porcine intestinum. For this purpose, different tissue fixation and staining methods that also allow potential subsequent immunohistochemical investigations were evaluated in the porcine mucosa, as well as submucosa of small and large intestine. Tissues were fixed in Carnoy, lead acetate, lead nitrate, Zamboni and ZSF and stained subsequently with either polychromatic methylene blue, alcian blue or toluidine blue. For the first time our study reveals that ZSF, a heavy metal fixative, preserves metachromatic staining of porcine MCs. Zamboni fixation was not suitable for histochemical visualization of MCs in the pig intestine. All other tested fixatives were suitable. Alcian blue and toluidine blue co-stained intestinal goblet cells which made a prima facie identification of MCs difficult. The polychromatic methylene blue proved to be the optimal staining. In order to compare MC counting results of the different fixation methods, tissue shrinkage was taken into account. As even the same fixation caused shrinkagedifferences between tissue from small and large intestine, different factors for each single fixation and intestinal localization had to be calculated. Tissue shrinkage varied between 19% and 57%, the highest tissue shrinkage was found after fixation with ZSF in the large intestine, the lowest one in the small intestine after lead acetate fixation. Our study emphasizes that MC counting results from data using different fixation techniques can only be compared if the respective studyimmanent shrinkage factor has been determined and quantification results are adjusted accordingly.

Keyword

mast cell, swine, fixation, tissue shrinkage factor

6.1. Introduction

Being strategically positioned at epithelial barriers, the mast cell (MC) takes on an essential role in intestinal mucosal surveillance.[1] MCs are well known for their role in allergy. Their ability to instantly degranulate and thus release soluble mediators from intracellular stores has been shown to be crucial for an optimal immune response.[2] Increasingly MCs become recognized as an important cell type mediating stress-related intestinal disorders,[1] but despite impressive progress, there are large gaps in our understanding of their phenotypic heterogeneity, of regulatory mechanisms involved and the functional significance.[3] Only recently it was revealed that immunological responses to probiotic bacteria may be MC mediated.[4] Probiotics as a dietary additive are claimed to improve the general health status of pigs, to reduce mortality and increase productivity rates, and are therefore in the focus of intense current research (*e.g*. recently reviewed in Cho *et al*.[5]), for example within a collaborative research center on intestinal microbiota-host interaction for which our laboratory provides a morphological profiling platform. The pig is also an important model for human intestinal physiology and pathology.[6] In this frame careful qualitative and precise quantitative investigations including subsequent histochemistry of MCs in the porcine intestine are necessary. Although histomorphological visualization, staining and counting of cells often is considered to be a trivial task, this is not true for MCs. One reason for this is that MCs represent a heterogeneous population. This heterogeneity, according to Moon *et al*., can be differentiated on the basis of their location, histochemical staining properties, their content of proteases [mostly determined by immunohistochemistry (IHC)], and reactivity to selected secretagogues and antiallergic drugs.[3] Initial research in the field of MC heterogeneity, including effects of fixation and staining, was done by Lennart Enerbäck. He described prominent differences for MCs concerning fixation sensitivity, morphology, membrane stability, granular content and dye binding properties between MCs situated in mucosal tissues and MCs found elsewhere, *e.g.* in the skin.[7-10] In pigs, mucosal-type MCs and connective tissue-type MCs have likewise been described due to histochemical properties.[11] However, it is postulated that heterogeneity of MCs in tissues is much more diverse than merely two polarized phenotypes and is probably dynamically changing in accordance with micro-environmental conditions.[3]

For clinical diagnosis and in research studies, various methods are used to stain MCs; in particular, metachromatic stains are frequently employed.[12-14] Several studies have shown that staining patterns of MCs, including the porcine ones, are critically dependent on the respective fixation technique employed.[11,15-17] Apparently, the detection of MCs in formalin fixed specimens is dependent on the species and the localization.[16,18-21] In the pig, mucosal and submucosal MCs did not stain or stained only faintly after formalin fixation[11,17] and a similar phenomenon of loss of metachromatic staining of mucosal MCs is seen in rats and humans.[15,22] There are some fixation methods that are particularly recommended for MC

staining, for example the fixation with Carnoy or lead salts.[15,16] One draw-back in characterisation of MCs by their histochemical as well as their IHC properties is that both techniques usually require different fixation techniques for optimal results, because fixatives conventionally employed for MC histochemistry do not allow optimal antigen preservation.[16] For example, in a study on intestinal morphology and mucosal immunity of piglets, Che *et al*. used Bouin's fixative for histochemical analysis including MC counts, and cryo-fixation for IHC analysis of immune cells.[23] Similarly, a study characterizing the intestinal mucosal immune cells of healthy young pigs used cryopreservation for RNAanalysis and Carnoy fixation for histochemical MC counting.[24] Shetye *et al.* compared the immunohistochemical reactivity of various leukocyte antigens in frozen and paraffinembedded tissues of rats, fixed in various fixatives. Antigen reactivity after fixation in Carnoy, 4% neutral buffered formalin and a mercury-based fixative (B-5) was not well preserved.[25] Similarly, the immunoreactivity of an antibody to ferritin was totally destroyed by Carnoy's fixative.[26] Of special interest is that Carnoy reduced the number of chymasepositive MCs in immunocytochemical staining of umbilical-cord-blood-derived human cultured MCs.[27] In contrast to this, Zamboni's fixative has been reported to be excellent for immunolabelling of MCs in the rat.[28] Another fixative, reported to be very effective for tissue preservation in different species and cell types, is zinc salt fixation (ZSF). It has been described to be clearly superior to formaldehyde, yielding results equal to cryopreservation;[29,30] still, to the best of our knowledge, it has never been used on MCs, let alone porcine ones.

In the light of the heterogeneous results on MC visualization published for different species, tissues and organs following the use of various fixation and staining methods, the aim of this study was to establish a reliable histological method for counting of MCs in the porcine intestinum. For this purpose different tissue fixation and staining methods that also allowed potential subsequent immunohistochemical investigations of MCs in the porcine intestine were evaluated. Tissues were fixed in Carnoy, lead acetate, lead nitrate, Zamboni's fixative and zinc salt. Stainings used included polychromatic methylene blue, alcian blue and toluidine blue. In order to compare MC counting results between the different fixation methods, tissue shrinkage was taken into account.

6.2. Materials and Methods

6.2.1. Sampling and tissues

Samples were taken in the course of a feeding trial, approved by the local state office of health and social affairs affairs, Landesamt, Berlin (LaGeSo Reg. Nr. 0347/09). National guidelines for the care and use of laboratory animals were followed. Twenty 39- to 55-day-old

conventionally kept German Landrace piglets were sedated with 20 mg/kg body weight (BW) of ketamine hydrochloride (Ursotamin®, Serumwerk Bernburg AG, Germany) and 2 mg/kg BW azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany) and then euthanized with an intracardial injection of 10 mg/kg BW tetracaine hydrochloride, mebezonium iodide and embutramide (T61®, Intervet, Unterschleißheim, Germany). Samples of mid jejunum and colon ascendens were collected from all animals and used for MC quantification. Samples of six piglets were additionally used for the determination of the shrinkage factor (see below).

6.2.2. Tissue processing

The samples were rinsed in ice-cooled Ringer solution, cut open on the mesenterial side and then trimmed to approx. 2×1 cm small squares, pinned loosely without stretching mucosal-side up on cork pieces and immediately fixed in the appropriate fixation solutions (Table 1).

Fixed tissues were dehydrated in graded series of ethanol, embedded in paraffin, cut to 5 µm thin sections, put on slides, de-waxed in xylene, rehydrated and stained according to the specific staining protocols. All tissue processing steps were carried out by the same experienced staff according to standardized protocols.

Table 6.2-1: **Table 1 - Standardized fixation procedures employed in this study, specifically adapted for the target tissue after fixation protocols indicated in the respective literature source.**

Fixation	Ingredients for 100ml	Fixation time and temperature	Notes
Carnoy[31,32]	60 ml ethanol, 30 ml chloroform, 10 ml glacial acetic acid	5-22 h 4°C	Dehydration starts with 100% ethanol – solution freshly prepared before use
Lead acetate[33,34]	4 g lead acetate x $3H_2O$ dissolved in bidistilled water ad 49 ml, 40 ml ethanol, 10 ml 37% Formalin, 1 ml glacial acetic acid	26 h room temperature	Dehydration starts with 70% ethanol
Lead nitrate[35,36]	8 g lead nitrate dissolved in 19 ml bidistilled water, 80 ml ethanol, 1 ml 37% Formalin	26 h room temperature	Dehydration starts with 80% ethanol
Zamboni[31,37,38]	2 g paraformaldehyde dissolved in 40 ml bidistilled water, 15 ml picric acid (saturated aqueous solution), 42.5 ml PBS (doubly concentrated), bidistilled water ad 100 ml	26 h 4°C	Fixation is followed by thorough rinsing in cold PBS (4°C)
Zinc salt fixation[39]	0.05 g calcium acetate 0.5 g zinc acetate 0.5 g zinc chloride, 1.2 g 0.1 M tris buffer, bidistilled water ad 100 ml	26 h 4°C or room temperature	Dehydration started with 70% ethanol

6.2.3. Histochemical staining

Polychromatic methylene blue modified according to Unna:[40]

- Deparaffinization and hydration to distilled water
- 1% polychromatic methylene blue in distilled water - 10 min (Methylenblau - Polychromes - Unna, Waldeck GmbH & Co., Division Chroma, Muenster, Germany)
- Rinse subsequently in distilled water, tap water, distilled water
- Differentiation in glycerine-ether (1:4 with distilled water) (Glycerinethermischung Unna, WALDECK GmbH & Co Division Chroma, Muenster, Germany)
- Rinse subsequently in distilled water, tap water, distilled water
- Drying in incubator 30 min at 60°C
- Quick immersion in 100% ethanol, xylene
- Coverslip with ROTI®-HISTOKITT (Carl Roth GmbH + Co. KG, Germany)

Toluidine blue and Alcian blue standard staining procedures.[35]

6.2.4. Tissue shrinkage

Tissue shrinkage was documented for every step of the embedding procedure. Samples were taken from 6 animals and two localizations (jejunum and colon) for each fixative employed. Tissue was laid mucosal-side up on filter paper (MN 616, circles, 185 mm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and directly cut on it. Each sample was placed together with the underlying paper in histology cassettes and put into the fixative. The contour of the tissue was marked on an extra sheet of filter paper (Whatman 10311810, grade 597, circles, 110 mm, Whatman plc, Maidstone, UK) after the fixation, the last ethanol step and the last paraffin step. The contour of the unfixed tissue was derived from the original paper cut. The marked filter papers were scanned; the areas labeled using the image manipulating software GIMP v2.6.11 (GNU Image Manipulation Program, http://www.gimp.org/team.html) and then measured with GSA Image Analyser v3.8.1 demoversion (GSA, Bansemer & Scheel GbR, Rostock, Germany). The shrinkage factor for every processing step (fixation, dehydration, paraffin embedding) was calculated as the rate of alteration of the area before (A_{fresh}) and after (A_x) a processing step with $f_{sample,x} = A_x / A_{fresh}$. Shrinkage was calculated as the decrease of area in percent after a processing step with $s_{sample,x} = (1-f_{sample,x})*100$.

6.2.5. Average shrinkage factor

The overall shrinkage factor after paraffin embedding $f_{sample,\ paraffin} = A_{paraffin} / A_{fresh}$ was used for further calculations. Average shrinkage factors $f_{fixation,\ localization}$ were calculated for each combination of fixation and localization for the normalization to fresh tissue.

Because Carnoy's fixative is frequently used for MC quantification, Carnoy shrinkage factors $fc_{fixation,\ localization} = f_{fixation,\ localization} / f_{carnoy,\ localization}$ were additionally used to normalize average shrinkage factors to this fixation.

6.2.6. Quantification of mast cells

Mast cells were manually counted in 4 different regions: jejunum mucosa, jejunum submucosa, colon mucosa, colon submucosa. For each fixation 3 slides were prepared and an area of at least 1.5 mm^2 was evaluated, employing the computer assisted image analysis program NIS-Elements AR (Nikon Instruments Inc., Melville, NY, USA). Concentration of MCs was measured for each sample as the number of MCs per area (MCs per $1mm^2$), $MCmm^2_{\ fixed} = MC / A_{fixed}$. To compare fixations, measurement values were normalized via the respective shrinkage factor to area of fresh samples with $MCmm^2_{\ fresh} = MCmm^2_{\ fixed} * f_{fixation,\ localization}$ or to Carnoy fixation with $MCmm^2_{\ Carnoy} = MCmm^2_{\ fixed} * fc_{fixation,\ localization}$.

6.2.7. Statistics

All calculations were performed with IBM SPSS 21 (IBM Deutschland GmbH, Ehningen, Germany). Effects on tissue shrinkage were assessed by a linear model with two fixed factors *localization* (colon and jejunum) and *fixation* (Carnoy, lead acetate, lead nitrate, Zamboni and ZSF) and with an interaction factor. Effects on MC counts were assessed by a linear mixed model with two fixed factors *localization* (jejunum mucosa, jejunum submucosa, colon mucosa and colon submucosa) and *fixation* (Carnoy, lead acetate, lead nitrate, Zamboni and ZSF), an interaction factor and with the factor *animal* (20 piglets) being a random effect. Post-hoc tests LSD (Least Significant Difference) were applied on fixed factors in case of statistically significant effects.

6.3. Results

6.3.1. Fixation of mast cells, histochemistry and morphology

Mast cells could be identified after fixation with ZSF, Carnoy, lead nitrate and lead acetate (Figure 1). The polychromatic methylene blue staining was characterized by its MC specific metachromatic staining in combination with low and uniform orthochromatic staining of the other components of the tissue. Co-stained goblet cells were a feature of both, alcian blue and toluidine blue staining, while the latter also displayed a considerable background staining. Concerning their morphology (Figure 2), porcine intestinal MCs were of oval to elongated shape in the submucosa and were found to have elongated or star shaped cell bodies in the mucosa. Compact, oval to roundish nuclei were visible in cells if not covered by granules. A varying amount of granules was found in the cytoplasm. These were particularly well preserved with the lead fixations that allowed their clear differentiation. In some of the samples fixed with Carnoy or ZSF the granules tended to dissolve and gave a blurry staining result (Figure 2). Also with the lead fixations in some samples veil-like metachromatic signals could be observed in the periphery of MCs (Figure 3A). Zamboni's fixative was not suitable for histochemical detection of MC in the porcine intestine (Figure 3B). In the submucosa of Zamboni-fixed samples some faintly stained MCs could be found but the granular content was not preserved and spread around the cells. Nearly no MCs were detectable in the mucosa.

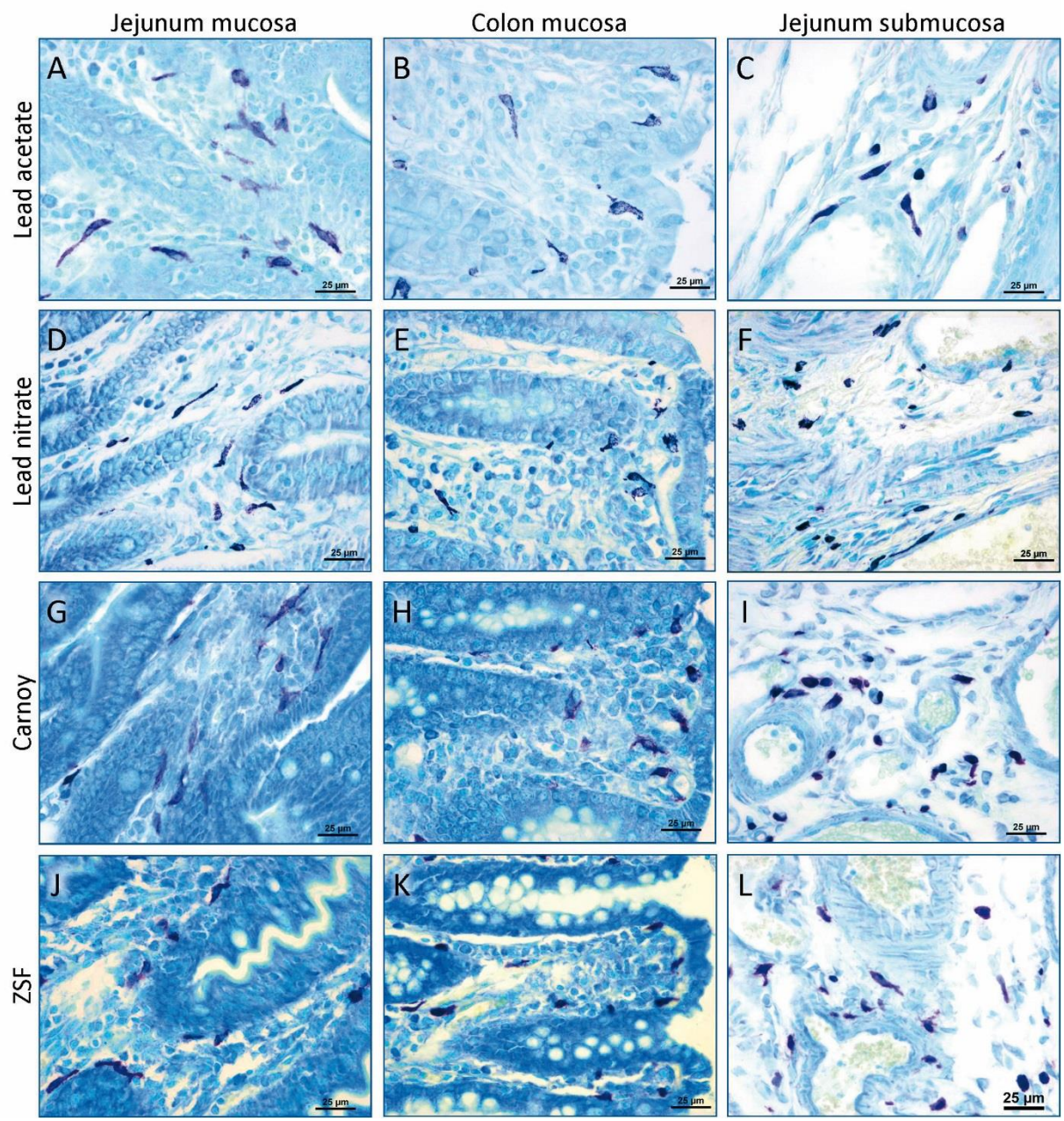

Figure 6.3-1: **Figure 1 - Histochemical demonstration of mast cells:**

A, B, C) Staining of jejunal and colonic tissue with polychromatic methylene blue after fixation in lead acetate. D, E, F) Fixation in lead nitrate. G, H, I) Fixation in Carnoy. J, K, L) Zinc salt fixation (ZSF). All samples were from the same animal and the pictures were taken with 400x magnification; scale bars: 25 µm.

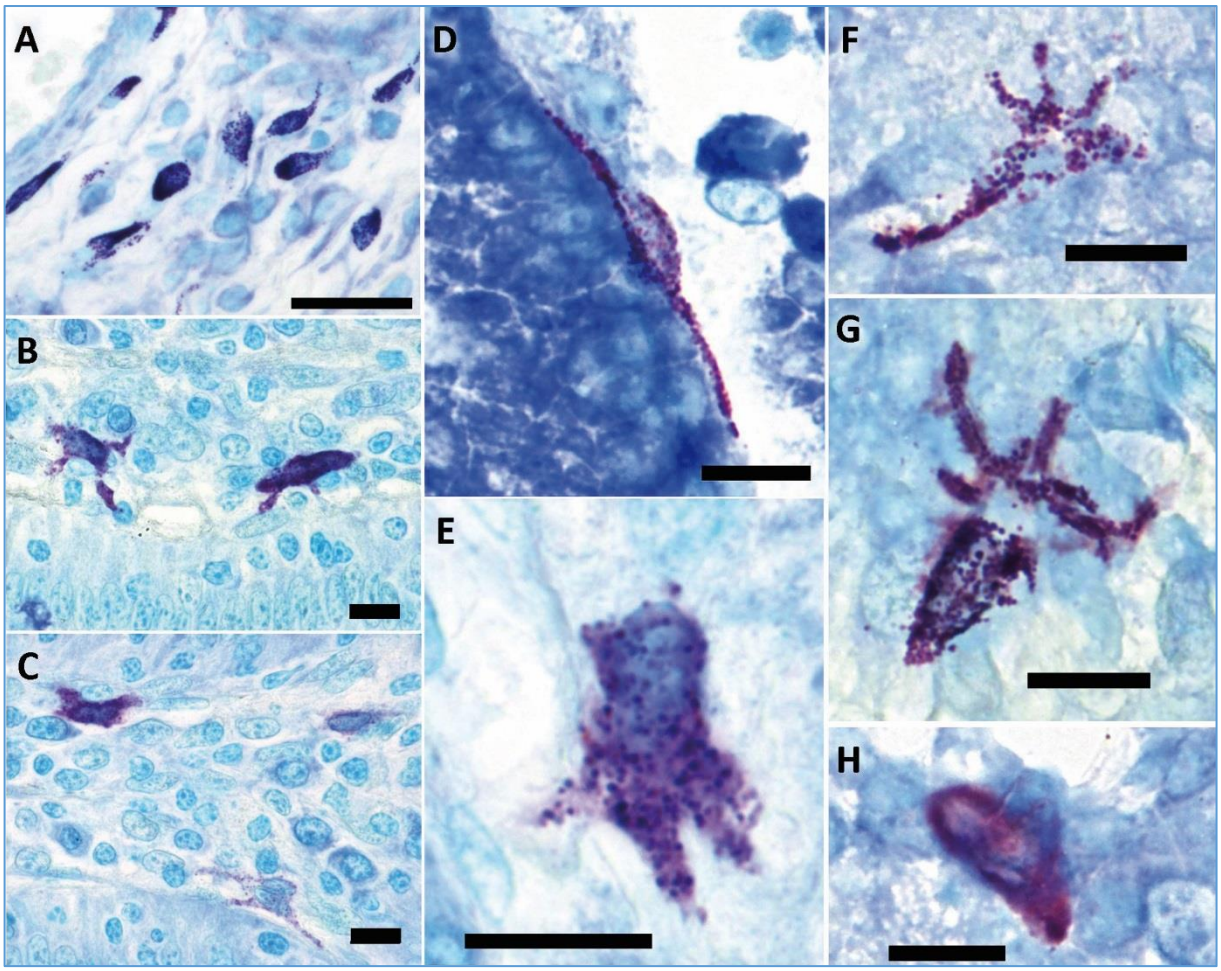

Figure 6.3-2: **Figure 2 - Morphology of porcine intestinal mast cells:**

Mast cells were of oval to elongated shape in the submucosa (A) and elongated or star shaped in the mucosa (B-G). A varying amount of granules could be found in the cytoplasm of MCs and compact, oval to roundish nuclei were visible in cells if not covered by granules (C). Morphological features and cytoplasmic granules were well preserved with the lead fixations (D-G), while MCs with dissolved granules were frequently found with Carnoy or zinc salt fixation (H). Tissues: colon submucosa (A), colon mucosa (F,G), jejunum mucosa (B-E, H); Fixation: lead nitrate (A, D, F, G), lead acetate (B, C, E), Carnoy (H); Staining: polychromatic methylene blue; scale bars: A, 25 µm; B-H, 10 µm.

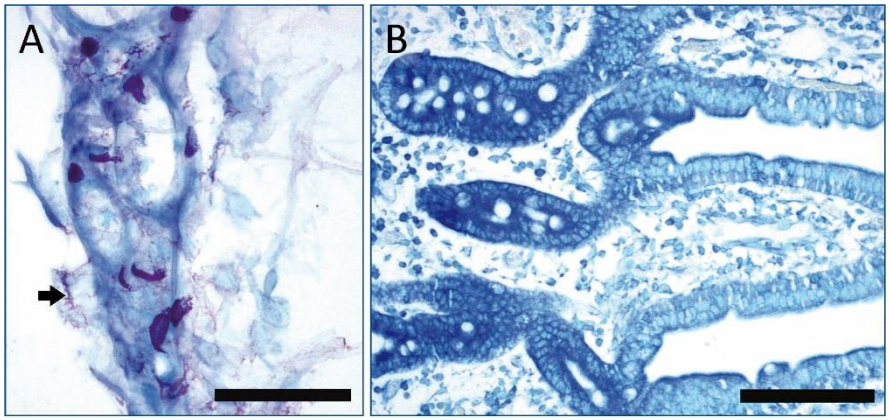

Figure 6.3-3: **Figure 3** A) Veil-like metachromatic staining signals in the periphery of mast cells of the jejunal submucosa (arrow) fixated with lead nitrate. They could be signs of a degranulation process due to the fact that granular content was otherwise precisely kept in place with that fixative. B) Metachromatic staining of mast cells was absent or faint in samples fixed with Zamboni. Scale bars: A, 50 µm; B, 100 µm.

6.3.2. Tissue shrinkage and resulting shrinkage factor

After paraffin embedding shrinkage has been found to vary between 19% (lead acetate - jejunum) and 57% (ZSF - colon) (Table 2). The shrinkage ratio calculated after the processing steps varied greatly between the fixations, and even within the same fixation differences between the small and large intestine were found. Resulting shrinkage factors varied significantly between fixations at both localizations (Table 3). Shrinkage factors varied also significantly for the localization after using the same fixation (Figure 4).

Table 6.3-1: **Table 2 - Shrinkage ratio for the processing steps (fixation, dehydration in ethanol and paraffin embedding) for the different fixatives in the small and large intestine.**

Fixation	***Tissue***	**Arithmetic mean of tissue shrinkage in percentage ± SD**		
		after fixation	*after ethanol*	*after paraffin embedding*
Carnoy	jejunum	19.46 ± 9.50	22.74 ± 11.37	41.33 ± 6.93
	colon	17.21 ± 7.48	19.62 ± 6.44	35.24 ± 5.14
Lead acetate	jejunum	4.77 ± 9.15	8.02 ± 8.93	19.21 ± 8.14
	colon	13.29 ± 8.96	15.06 ± 10.19	25.61 ± 8.82
Lead nitrate	jejunum	24.52 ± 8.92	24.02 ± 9.19	33.21 ± 7.80
	colon	34.20 ± 7.81	36.23 ± 7.45	43.15 ± 6.84
Zamboni	jejunum	9.07 ± 7.48	30.39 ± 6.99	41.13 ± 5.66
	colon	11.47 ± 3.23	27.67 ± 4.49	39.64 ± 4.77
Zinc salt fixation	jejunum	35.53 ± 7.35	36.67 ± 6.84	42.91 ± 7.28
	colon	48.89 ± 7.11	50.63 ± 6.53	56.53 ± 5.43

Table 6.3-2: **Table 3 - Arithmetic mean of resulting shrinkage factors and significance groups for fresh tissue with confidence intervals and for normalization to Carnoy.**

Arithmetic mean of resulting shrinkage factors and significance groups					
	Carnoy	**Lead acetate**	**Lead nitrate**	**Zamboni**	**Zinc salt fixation**
Jejunum	0.59 (0.53-0.64)	0.81 (0.75-0.86)	0.67 (0.61-0.72)	0.59 (0.53- 0.65)	0.57 (0.52-0.62)
	1.000	1.377	1.138	1.003	0.973
SG	A	C	B	A	A
Colon	0.65 (0.59-0.70)	0.74 (0.69-0.80)	0.57 (0.51- 0.62)	0.60 (0.55-0.66)	0.44 (0.38-0.49)
	1.000	1.149	0.878	0.932	0.671
SG	C	D	B	B,C	A

[i] SG, significance groups. A, B, C, D, different letters indicate for significant differences among fixations; same group for two fixation means there is no significant difference between them.

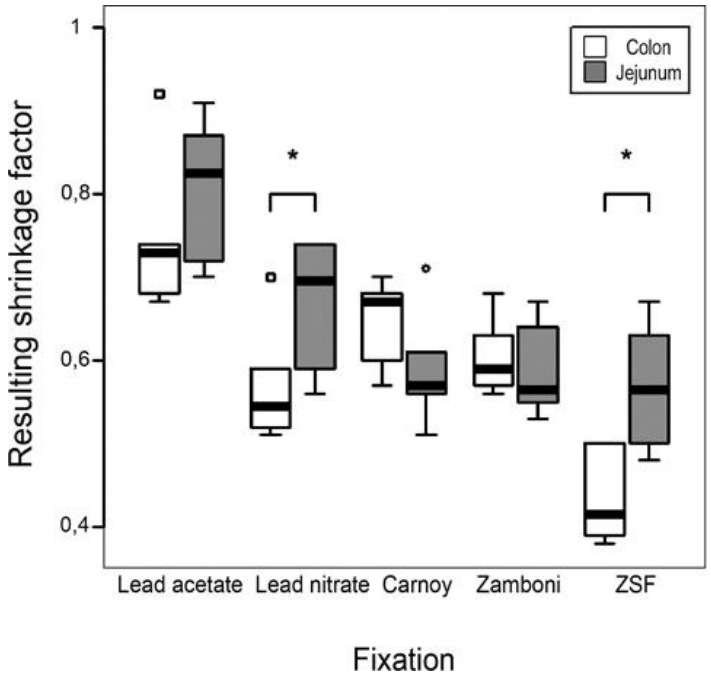

Figure 6.3-4: Figure 4 - Comparison of the resulting shrinkage factors for colon and jejunum; the shrinkage varied between localizations after using the same fixation. Carnoy and Zamboni showed no difference whereas shrinkage of colonic tissue was increased compared to jejunal tissue in the heavy metal salt fixations. The effect was significant (indicated by asterisks) for lead nitrate and zinc salt fixation (ZSF).

6.3.3. Quantification and statistics

Zamboni was not suitable for counting MCs via metachromatic staining and resulted only in a fractional amount of visible MCs in the submucosa and nearly no MCs in the mucosa compared to the other fixations. Results from tissues fixed this way were therefore eliminated from further analysis. MC counts were adapted via the shrinkage factors (Figure 5). In jejunal mucosal tissue, MC numbers were consistent in all examined fixations after normalization. For other tissue sections however, various significance groups could be found (Table 4). If no normalization factor was applied, the significance groups showed a different distribution pattern (Table 4).

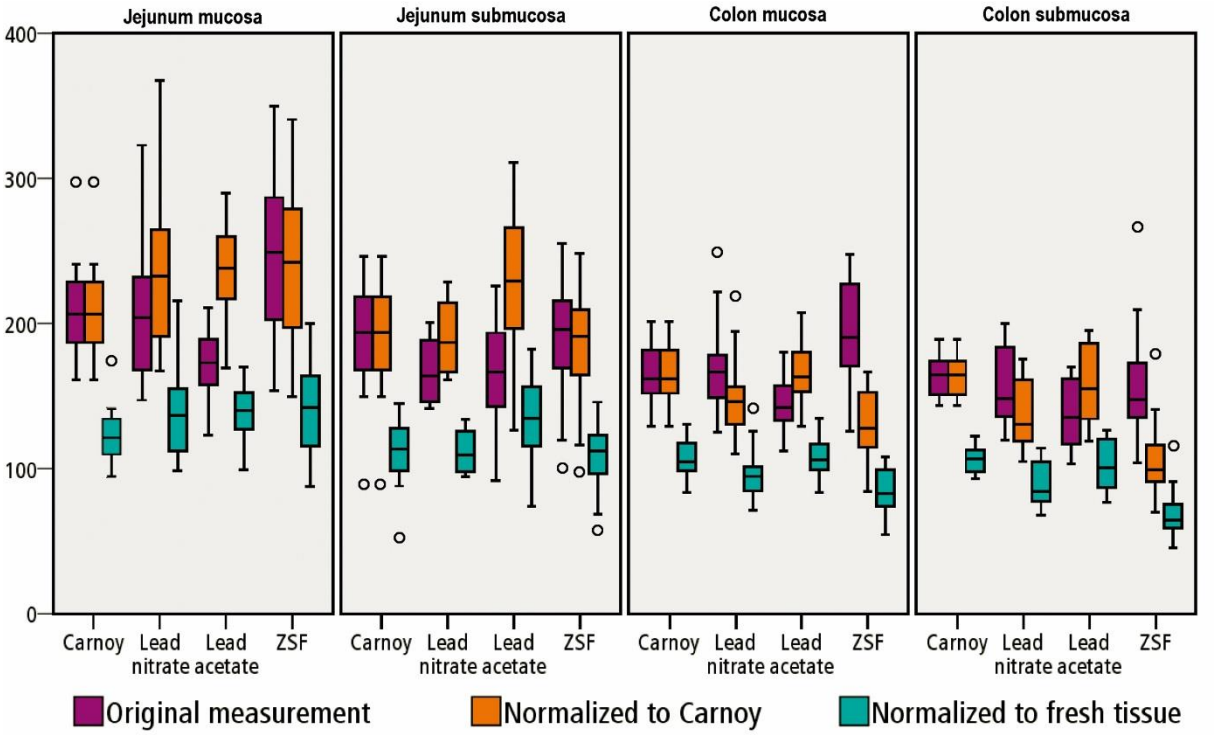

Figure 6.3-5: Figure 5 - Mast cells per mm²:

Original mast cell counting results compared with results adapted via the shrinkage factors individually plotted for each tissue section.

Table 6.3-3: Table 4 - Number of mast cells and differences between original and normalized data: Mast cells numbers with confidence intervals in this table were normalized to Carnoy fixation.

Number of mast cells and differences between original and normalized data				
	Carnoy	**Lead acetate**	**Lead nitrate**	**Zinc salt fixation**
Jejunum mucosa	217 (195 - 240)	238 (217 - 258)	239 (219 - 260)	236 (219 - 253)
SG Carnoy	A	A	A	A
SG original	B	A	B	C
Jejunum submucosa	193 (171 - 215)	229 (207 - 251)	190 (170 - 211)	184 (167 - 202)
SG Carnoy	A	B	A	A
SG original	A	A	A	A
Colon mucosa	173 (151 - 195)	167 (147 - 188)	149 (129 - 170)	129 (112 - 147)
SG Carnoy	B	B	A, B	A
SG original	A, B	A	A, B	B
Colon submucosa	170 (147 - 192)	160 (139 - 180)	138 (118 - 159)	105 (87 - 122)
SG Carnoy	C	B, C	B	A
SG original	B	A	A, B	A, B

[i] SG, significance groups. SG original, distribution of groups for the original measurement without normalization. A, B, C, D, different letters indicate for significant differences among fixations; same group for two fixation means there is no significant difference between them.

6.4. Discussion

The aim of this study was to evaluate tissue fixation and staining methods for subsequent identification of MCs in the porcine small and large intestine. Tissues were fixed in Carnoy, lead acetate, lead nitrate, Zamboni and ZSF and stained subsequently with either polychromatic methylene blue, alcian blue or toluidine blue. Results showed that Zamboni fixation was not suitable for histochemical visualization of MCs in the porcine intestine. All other tested fixatives were suitable for histochemical visualization of porcine intestinal MCs, however, MC counts in the submucosa of the small intestine and in the mucosa and submucosa of the large intestine were heterogeneous. In order to compare MC counting results of the different fixation methods, tissue shrinkage was taken into account. The highest tissue shrinkage was found after fixation with ZSF in the large intestine, the lowest one in the small intestine after lead acetate fixation.

6.4.1. Fixations

Staining properties of MCs are influenced by the method of fixation. Our results showed that all fixatives tested were suitable for histochemical visualization of porcine MCs with the exception of Zamboni's fixative that did not preserve metachromatic staining properties of MCs, neither in the porcine intestinal mucosa nor submucosa. In contrast to our results, Zamboni was reported to preserve the granules of MCs well and distinctly in the rat, as shown by both light- and electron-microscopic IHC.[28] This highlights the species-specific properties of MCs and emphasizes the influence of fixation procedures on MC detection. An earlier study[41] describing MC staining in the camel also underlined the fact that data regarding MC heterogeneity from other species, obtained by different fixation methods, are not comparable. A reason for the species-specific reaction of MCs to specific fixatives is probably the different amount and combination of chemical constituents within their cytoplasmic granules.[42]

Jirge *et al*. studied MCs histochemically in relation to various fixation and staining procedures in the pig and other species. Similar to the results of our study, they described that counts of MCs in the pig were higher after heavy metal fixation than after fixation with aldehydes, but *vice-versa* in other tested species.[43]

Like the aldehyde and heavy metal fixations, Carnoy is also frequently used for the examination of MCs[17,20,44-47] and in our study has been found to show satisfactory results for the evaluation of porcine MCs. This fixative, which contains ethanol, chloroform and glacial acetic acid, is suitable for the histochemical examination of proteins and carbohydrates and has been used in many conventional and special staining techniques.[49] Granules of MCs in special locations (*e.g.,* mucosal MCs) in many species are water soluble and therefore require alcoholic fixation (like Carnoy) for preservation.[49] Likewise, lead acetate and lead nitrate both contain alcohol plus a heavy metal salt. Parwaresch and Lennert found that the complete water

resistance of basophilic granules manifests their sufficient fixation and that for example basic lead acetate proved to be a good fixative for that purpose.[50] The mechanism behind this is likely to involve the precipitation of mucopolysaccharides or associated proteins from the MC granules.[7]

Focus has turned recently to a certain nonaldehyde based fixative, *i.e*. ZSF. It combines good morphological with good antigen preservation including intracellular and surface epitopes and for example allows simultaneous analysis of DNA content and synthesis along with the analysis of cells via flow cytometry.[29,51-53] Therefore we also included zinc salt as a new fixation procedure for MCs. For the first time our study revealed that ZSF, also a heavy metal fixative, preserves metachromatic staining properties of porcine MCs. Barranco *et al*. compared three different fixatives, namely 10% buffered formalin, Bouin and ZSF, and found only the latter one to be an excellent fixative for IHC in apoptosis assays for porcine tissues.[54] ZSF was also used to detect immune system cells in mice[29] and humans[55] with good results. Moreover, a considerable advantage of ZSF compared to the other heavy metal fixatives as well as Carnoy fixative is its reduced toxicity.

6.4.2. Histochemistry and morphology

Mast cells were first described by Paul Ehrlich in his 1878 doctoral thesis, on the basis of their large granules and unique socalled metachromatic staining.[56]

As the MC-specific granules contain the glycosaminoglycans heparin and/or chondroitin sulfate, *i.e.* sulphated mucopolysaccharides which are responsible for their metachromatic labeling,[9,10,57] it is important to use a fixative which preserves these components. In our study, the metachromatic dyes toluidine blue and polychromatic methylene blue as well as the orthochromatic alcian blue were tested for MC staining. Alcian blue co-stained intestinal goblet cells, which made a *prima facie* identification of MCs difficult within our target sample tissue. Toluidine blue likewise stained goblet cells and produced a considerable background staining. Polychromatic methylene blue staining was superior to alcian blue and toluidine blue due to its clear MC-specific metachromatic staining in combination with low and uniform orthochromatic staining of the remaining compounds of the tissue. This result from our study is also supported in earlier evaluations of different histologic stains for MCs in primates, guinea pigs and dogs.[58,59] Nevertheless, the variable, presumably tissuespecific staining behavior of MCs has generally to be taken into account even when comparing results from one species, as for example Xu *et al.* found a significant difference when comparing porcine MC counting results using the same fixation but different stainings. They compared alcian blue and toluidine blue and found the first one to stain more MCs,[17] which was also confirmed by Vodenicharov *et al.* for the porcine ureter.[60] In general, intestinal MCs have a lower affinity to thiazine dyes like toluidine blue and a higher affinity for basic copper phtalocyanine dyes like alcian blue.[8]

It has to be kept in mind that, similar to MCs, basophils also contain prominent metachromatic cytoplasmic granules;[61] but it was demonstrated that cells of the basophilic cell line contribute to no great extent to the number of metachromatic cells in normal intestinal mucosa.[62] By counting granulocytes and MC of the intestinal mucosa of probiotictreated and control piglets, Duncker *et al*. found no basophils within the mucosal layers in untreated animals.[24] Therefore, stainingconditioned erroneous inclusion of basophils in either digital analysis system assisted or (as in our study) manual MC counts are probably not of great importance when assessing healthy tissue. Counting of MCs asks for a reliable staining and identification. Since the times of Ehrlich the majority of identification methods for MCs depend on the presence of components of secretory granules which disappear upon degranulation. As Buckley and Walls reviewed, in most cases MC degranulation may be partial rather than total, but there may be particular difficulties in detecting MCs where a substantial degree of degranulation has occurred. The potential for such *phantom MCs,* which fail to stain with dyes is well established.[16] Therefore, we cannot exclude that also with the carefully adapted and evaluated procedures employed within our study, the recorded MC number may have been underestimated.

Mast cell morphology was found to be in accordance with descriptions from the literature.[17,49,60,63,64] An interesting finding were the star shaped MCs which resided in the mucosa of both jejunum and colon and were not mentioned in the description of porcine MCs by Xu *et al.*[17] The star shaped MCs and also the bigger spindle shaped MCs with elongated processes represent presumably an activated or special type of MC whereas the smaller round cells represent a resting state.[65-67]

6.4.3. Shrinkage

Tissue shrinkage is an important factor with a crucial impact when comparing results gained from differently processed samples.[68] In order to allow comparison of MC counting results published by other authors and from own data, the respective study-immanent shrinkage factor needs to be determined and quantification results have to be adjusted accordingly.

In our study we found that shrinkage after paraffin embedding varied between 19% and 57%, a fact which obviously had great impact on the MC quantification results. The uncorrected data for jejunum mucosa implied differences in counting results between the different fixatives employed, whereas after correction these differences were no longer present. The shrinkage ratios after the different processing steps (fixation, alcohol dehydration and paraffin embedding) varied; *i.e.,* after lead acetate fixation of the jejunum shrinkage ratio was 4.77±9.15%, *e.g.* in contrast to 48.89±7.11% after ZSF fixation of colonic tissue. In addition to the variations of tissue shrinkage employing the different fixations, even using the same fixation

caused shrinkage-differences between tissue from small and large intestine. This demanded the use of different factors for each single fixation and intestinal localization.

Clearly there are limits to the method applied in our study, in which only the areashrinkage of the whole tissue was measured. Earlier studies on effects of histological processing in cancer samples[69] reported that variables like whole-mount *vs* quadrant sections, thickness of tissue slices, length of time of the alcohol dehydration steps, and varying temperature of the flotation bath did not have a significant influence on tissue shrinkage. Within our study we observed that mucosa and submucosa seemed to shrink to a different extent and not evenly in all samples. Therefore intraorgan specific differences between mucosa and submucosa can be assumed, potentially influencing the results. Architecturally, the mucosa is a relatively *tough* compartment in contrast to the underlying submucosa which is designed to act as a shifting layer between the tunica mucosa and the tunica muscularis, enabling them to move against each other,[70] possibly resulting in varying shrinkage potential for this compartment. In a study on porcine stomach submucosa an individual variation in a range from 3 to 70% of the observed morphological parameter was detected.[71] Their and our results indicate that in future studies on (mast) cell counting individual shrinkage factors for individual tissue layers may need to be determined.

6.4.4. Mast cell quantification results of porcine intestinal tissue

Strobel *et al.* emphasized that differences in fixation and staining techniques may explain the contradictory results in the published reports on the number of human MCs in the gastrointestinal mucosa in health and disease. Their results show a critical influence of the fixative on the number of MCs identified after staining. These authors discussed the tissue shrinkage, but decided that it would not bias their results.[15] When comparing available data concerning the pig from studies employing the same fixatives as tested within our study, our results from MC counting were generally in agreement with the results of other authors.[11,12,17]

Besides the fixation, a limiting factor for the comparison of results of different studies is the variety of used material and methods. Moreover, age[72] and breeding conditions may also influence the number of MCs. The incidence of (sub-)clinical conditions such as infection with parasites *etc*., has a well-known impact on MC numbers.[11,73-75] Therefore, comparison of data from studies using different tissue processing procedures should be approached with caution, because without a corrective tissue shrinkage factor these data may not objectively be transferable.

6.5. Conclusions

Tissue shrinkage is an important factor with a crucial impact when comparing results gained from samples processed with different fixatives. Thus, in order to allow comparison of data, the respective study-immanent shrinkage factor needs to be determined and quantification results have to be adjusted accordingly. Polychromatic methylene blue is suitable for the detection of porcine intestinal MCs when their granular content is properly fixed. ZSF - a new fixative recently employed for its excellent antigen-preservation properties used in IHC - is suitable for detecting MCs via histochemical methods. ZSF may therefore be the fixative of choice for studies planning to characterize MCs by both histochemical and immunohistochemical methods that hitherto demanded the combination of different fixation procedures for optimal results.

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7. Discussion

The objective of this thesis was to further our knowledge on the effect of *E. faecium* on the complex processes associated with nutritional interventions in the intestinal mucosal network. The focus was laid on the mucosa, since this component was identified as an important crossroad where nutrition, microbiota, pathogens and host meet. A broad spectrum of histological methodologies was refined and applied to the porcine organism in the course of the described trials. The effects of different nutritional strategies on histological parameters under normal housing conditions or in challenge situations were examined.

Three different main publications resulted from this thesis, reporting on mucosal parameters in probiotic (*Enterococcus faecium* NCIMB 10415) treated piglets in a challenge situation with *Salmonella* Typhimurium, on the immunohistochemical detection of *Salmonella* Typhimurium in porcine mucosa associated tissues and on the histochemical detection of porcine mucosal and submucosal mast cells.

A particularly important aspect of the research work was the joining of histological knowledge with additional methodological approaches in team with researchers of the SFB 852. By joining branches of the field of biomedical sciences (e.g. nutrition, virology, cell culture, physiology and immunology) a picture of the processes in intestinal mucosal network was gathered in more detail.

Most parameters reported on in chapters 4-6 were studied in young animals. Therefore an enlightening view on the developmental aspects of the intestinal mucosal network was possible.

In the following, 3 focal points concerning the intestinal mucosal network and important issues in histological studies on nutrition-microbiota-pathogen-host-interactions will be discussed: developmental changes (7.1), nutrition related changes (7.2) and methodological aspects (7.3).

7.1. The intestinal mucosal network: Developmental changes and the influence of weaning

Young pigs around weaning are especially prone to gastrointestinal dysfunctions caused by a multitude of stressors. This situation provokes a growth delay which can be seen on a macroscopic level as reduced daily weight gain (Lallès *et al*, 2007). On a microscopic level the intestinal mucosa shows changes due to this stressfull event. Al Masri et al. (2015) recently reviewed the morphological changes associated with normal age dependant development, artificial weaning and different feeding regimes in the porcine small intestine. Natural weaning occurs around week 17 in domestic pigs and villi undergo shortening before, which is associated with a decline in nutrient content of the sow´s milk. Immediately after weaning a

rapid reduction in villus height takes place up to a maximal atrophy of 60% between 3 and 5 days after weaning. From there villus height slowly increases, not reaching pre-weaning values. Since villus height also decreases in natural weaning, this can be viewed as part of the small intestine's normal development (Al Masri *et al*, 2015). This demonstrates that age has a huge impact on morphometric data, especially around weaning and comparable results were found when the parameter "age" was investigated in one of the studies for this thesis:

In the study of Rieger et al. (2015a) (chapter 4), the performance of probiotic (*Enterococcus faecium* NCIMB 10415) treated animals was to be examined in a challenge situation with *Salmonella* Typhimurium. Therefore the jejunal mucosal surface available for nutrient absorption was determined morphometrically. It was observed that age / time post infection had a major influence on the respective parameters (villous length, crypt depth, villus enlargement factor and ratio between villus and crypt enlargement factors), the older animals showing higher values (Rieger *et al*, 2015a). In conclusion it is important to note that morphometric data like villous height and crypt depth always have to be placed, analysed and interpreted in the developmental context.

The same principle seems to be true for the evaluation of other mucosal parameters. Unpublished results from the study of Rieger et al. (2013) (chapter 6) showed the possible influence of age on the number of mucosal mast cells (Rieger *et al*, 2013). Employing and refining the methods described in chapter 6 for the study by Kröger et al. (2015), the number of colonic mucosal mast cells was found to be age dependant, increasing with age (Kröger *et al*, 2015). Age / time post infection had also a major influence in the study of Rieger et al. (2015a) (chapter 4) on the number of jejunal intraepithelial lymphocytes - older animals showing an increased number. This was interpreted as an age driven process as well as an infection driven one and is in agreement with the observation made by Solano-Aguilar et al. (2001) who demonstrated that the age of the pigs as well as weaning have a significant effect on the number of lymphocytes isolated from e.g. ileal mesenteric lymph nodes (Solano-Aguilar *et al*, 2001).

This again underlines the necessity that such data always has to be placed, analysed and interpreted in the developmental context.

7.2. The intestinal mucosal network: Nutrition related changes

7.2.1. Effects of *Enterococcus faecium*

Since immunoglobulins are not able to cross the porcine placenta, newborn pigs cannot mount prompt systemic or mucosal immune responses. It is imperative for them to acquire maternal immunity, first via colostrum and then via milk. Within the first 36 h of life, the piglet has to absorb maternal IgG from the colostrum to acquire humoral immunity. Secretory IgA for passive mucosal immunity will be supplied via the milk until weaning. Also cellular immunity via maternal cells is transferred via colostrum and milk. Interestingly, the colostrogenic immunity is dependent on the systemic immunity and the lactogenic immunity depends on stimulation via induction sites (gut and respiratory tract - described in section 2.3.1) (Salmon *et al*, 2009). In general, the mucosal immune system seems to remain relatively immature throughout the early weaning period and for example intraepithelial lymphocytes were shown to have a transient reduction of reactivity (Lallès *et al*, 2007).

Recently it was shown that intraepithelial lymphocytes are under a continuous selection pressure, enforced by the epithelium. This selection enriches intraepithelial lymphocytes with a high-avidity/-affinity for long-term survival, whereas less antigen-sensitive cells are constantly eliminated from the pool of resident mucosal effector memory T cells. Many of them are thus highly sensitive and fully differentiated antigen-experienced memory T-cells, which function as effective sentinels and rapidly respond to pathogens (Cheroutre & Huang, 2012).

Consequently, in the *Salmonella* challenge study of Rieger et al. (2015a) (chapter 4), a point of interest was the diverse population of intraepithelial lymphocytes as a component of the epithelial barrier. Concerning the probiotic treatment, several effects were found. The probiotic treated animal group generally had higher numbers of intraepithelial lymphocytes. The differences for nuclear level intraepithelial lymphocytes presented the most prominent quantitative contrasts and separate analysis of age groups revealed that significant differences were only found at day 28 post infection. A minor advantageous effect on the mucosal morphometric data (tendentially longer villi and a higher ratio of villus enlargement factor/crypt enlargement factor 28 days post infection) could also be attributed to the probiotic treatment.

It is known that intraepithelial lymphocytes are involved in epithelial tissue homeostasis. They monitor neighbouring epithelial cells for signs of damage or disease. They can lyse damaged or malignant epithelial cells, recruit inflammatory cells to the site of damage and directly mediate epithelial repair and regeneration via secretion of epithelial growth factors, e.g. keratinocyte growth factor (Havran *et al*, 2005; Meehan *et al*, 2014). Therefore it was concluded that the intraepithelial lymphocytes positioned at the nuclear level of the epithelium possibly had a probiotic related positive influence on the epithelium, resulting in improved mucosal morphological features at 28 days post infection. Moreover it was concluded that

intraepithelial lymphocytes at the nuclear level might be of great functional importance in piglets and involved in protection of epithelial integrity.

Besides the already described results, *E. faecium* supplementation provoked a variety of immunological responses as reported in other published studies from our research group: enhanced innate responses due to stimulation of pro-inflammatory cytokines IL-6 and IL-8 in a challenge with transmissible gastroenteritis coronavirus (Chai *et al*, 2013); increased humoral immune responses due to higher specific antibody levels in a challenge with swine influence virus (Wang *et al*, 2014); lower Th2 associated immune response in a *Salmonella* Typhimurium challenge (Kreuzer *et al*, 2014).

In the last mentioned study of Kreuzer et al. (2014) the influence of *E. faecium* on CD25+ and other T helper cell subsets was analysed in the ileum and mesenteric lymph nodes. CD25 is the alpha chain of the trimeric high affinity IL-2 receptor. It is essentially expressed on activated T cells and T regulatory cells (Tregs). Tregs control the functions of immune competent effector cells, for example CD8+ cytotoxic T cells. They are classified as natural (response to autoantigens) or induced (response to exogenous antigens) Tregs and they produce TGFß and IL10 (anti-inflammatory cytokines). The effect of *E. faecium* on Tregs in the course of a challenge trial is of interest, because pathogens can escape immune response via Treg induction (Silva-Campa *et al*, 2012); bacteria can induce IL-10 and TGF-β in Tregs (Round & Mazmanian, 2009); TGF-β from Tregs depresses antigen specific proliferation of naïve CD4+ T cells (Kubiczkova *et al*, 2012); *E. faecium* induces IL-10 and TGF-ß (Huang *et al*, 2012) and reduces CD8αβ T cells (Mafamane *et al*, 2011). In our study, no significant differences in presence and quantity of CD25+ cells and amount of other T helper cell subset related transcription factors were found in intact tissue samples. In contrast, after magnetic sorting of T helper cells, transcription factors in the lymph nodes of probiotic treated piglets were found to be significantly different from the control group and a more effective immune response was suggested for the control group.

Interestingly, *E. faecium* seemingly enhanced immune reactions against virus infections but had an adverse effect in *Salmonella* infection. As stated by Bailey (2009), probiotics shall optimize the immune system and changes of immunological measurements can not be interpreted on their own (Bailey, 2009). Consequently, the results of the above mentioned studies had to be put in a bigger context and more studies investigating the influence of *E. faecium* were reviewed for immunomodulatory effects as well as piglet performance. Siepert et al. (2014) commented about several studies with the probiotic and it was noticed that *E. faecium* supplementation, in combination with a *Salmonella* challenge, reduced intestinal immune-associated gene expression in post-weaning piglets. They concluded that this may delay the host response to infections, "*and provide pathogenic bacteria such as Salmonella with a "window of opportunity", leading to the increased bacterial loads and shedding observed*

in challenge trials" (Siepert *et al*, 2014). Twardziok et al. (2014) analysed a comprehensive data-set from the feeding experiments with *E. faecium* in the frame of the SFB852. Their observations appear to support the idea of the potential immuno-modulatory effects. They give two possible explanations: "*probiotic supplementation might alter the composition of the intestinal community, such that it relates less to the host immune system; or alternatively, the supplementation dampens the response of the immune system to variations in the intestinal communities*" (Twardziok *et al*, 2014). The European Food Safety Authority recently published a scientific opinion on the safety and efficacy of *E. faecium* as a feed additive for pigs. The additive, which is attributed the function of a gut flora stabiliser, is considered safe for pigs, consumers, users and the environment. Interestingly *E. faecium* was deemed to have the potential to improve one or more performance parameters of sows and of suckling piglets as well as to be efficacious in weaned piglets (EFSA FEEDAP Panel, 2015). In a study by Broom et al. (2006), supplementation of the post-weaning diet with *E. faecium* did not affect piglet performance and *E. faecium* did not affect gastrointestinal bacterial populations but did tend to reduce serum IgG. Thus, dietary supplementation with *E. faecium* was deemed to be ineffective under the particular trial conditions (Broom *et al*, 2006).

In summary the use of *E. faecium* as a probiotic may seem to have ambivalent effects on the mucosal immune system and performance. It was demonstrated to show beneficial as well as undesirable effects or no influences at all. I propose that the probiotic should be used targeted and the animal herd to be treated should be monitored for e.g. *Salmonella* infection in which case a supplementation seems not to be beneficial. Nonetheless, as the results from the virus experiments are promising, more research on the specific mechanism of action is necessary. Also the effect of the probiotic on intraepithelial lymphocytes is of importance. A phenotypic characterisation of the intraepithelial lymphocytes *in situ* would be crucial to identify whether the ones at the nuclear level represent a special functional subset (also commented on further down). The overall immuno-modulatory effects of the probiotic promise to be valuable if applied in the right setting, for example in a condition like intestinal bowel disease, where a balanced mucosal immune response is advantageous. An interesting research question could adress the interaction between mast cells and probiotics regarding the regulation of intestinal mucosal barrier function. It was found that persistent life stress and stressful life events affect the course of irritable bowel syndrome and intestinal bowel disease through largely unknown mechanisms and that a positive effect of probiotics could involve mucosal mast cells and their function in epithelial barrier permeability regulation (Carlsson, 2013).

7.3. The intestinal mucosal network: Methodological aspects

7.3.1. Histological methodologies applied to improve characterization of microorganisms and immune cells

7.3.1.1. Microorganisms

It was emphasized by Campisi et al (2008) that imaging studies play a critical role in the understanding of the host's immune system response to a pathogen like *Salmonella* (Campisi *et al*, 2008). Diagnostic immunohistochemistry is a valuable technology, e.g. in surgical pathology, and the precise methodological details (fixation, processing) that are used have a major effect on final results (Miller *et al*, 2000).

The study of Rieger et al. (2015b) (chapter 5), which was performed in the same trial like the one of Rieger et al. (2015a) (chapter 4), focused on the immunohistochemical detection of *Salmonella* Typhimurium in porcine mucosa associated tissues. The aim was to visualize the *in situ* localization of the bacterium in addition to e.g. bacterial quantification in homogenized tissues. Although *Salmonella* was detected with the immunohistochemical protocol used, some technical finesse proved to impressively improve the sensitivity of the test (Rieger *et al*, 2015b). It can be concluded that, although methods like bacterial plate counting or molecular biological methods should still be the main path to quantify bacteria in a certain tissue compartment, immunohistochemistry can be used to estimate the degree of infection and simultaneously to rank the pathological alterations and localize the exact way *Salmonella* takes on its route through the body.

7.3.1.2. Lymphocytes

As already mentioned above, a phenotypic characterisation of intraepithelial lymphocytes *in situ* would be very advantageous. The planned immunohistochemical phenotypic characterisation of intraepithelial lymphocytes was not possible due to a lack of suitable antibodies for paraffin embedded specimens (which were the only ones available from this trial). Much effort was invested to establish staining protocols, unfortunately without success (data not published). Later this task was tackled again in a separate project for a master student (Schulte, 2015) - with the same results. As already described by Hicks et al. (2006), aldehyde based fixatives often cause a loss of epitope recognition during fixation due to crosslinking mechanisms. The failure to preserve lymphocyte epitopes was in this case demonstrated for samples of murine origin (Hicks *et al*, 2006). In conclusion, so far - a suitable antibody-panel to characterize porcine intraepithelial lymphocytes in paraffin embedded

specimens is not available. Therefore the intraepithelial lymphocytes were quantified *in situ* with histochemical methods.

Another aspect of the above mentioned animal studies (Rieger et al. 2015a and 2015b) was examined in the experiment of Kreuzer et al. (2014). The influence of *E. faecium* on CD4+ T helper cells was analysed in the ileal mucosa and associated lymph nodes. On the one hand it was demonstrated that immunohistochemistry is excellent for *in situ* localization of CD25+ cells and their morphological profiling. On the other hand, it was shown that additional methods like flow cytometry enable effective quantification and analysis of the cells. This is in agreement with Gerner et al (2012), who favour flow cytometry for quantitative analysis of complex cell populations but also stress the failure of this method to get information on tissue localisation for which they would use histology. As a solution, an analytical microscopy method, "histo-cytometry," for visualizing and quantifying phenotypically complex cell populations directly in tissue sections is described. The authors state to be able to "*identify complex cellular subsets and phenotypes, achieving quantitatively similar results to flow cytometry, while also gathering cellular positional information*"(Gerner *et al*, 2012).

In agreement with Gerner et al. (2012) I am convinced that combining flow cytometry and immunohistochemistry will be the method of choice in order to gather precise and reliable information on quantity and distribution of cellular subsets in a complex network like the mucosa or other tissue compartments and organs.

7.3.1.3. Mast cells

The study of Rieger et al. (2013) (chapter 6) focused on porcine intestinal mast cells, an important cell population in regulating intestinal barrier function and host defence. A histochemical protocol was established to reliably quantify mast cells in porcine tissues and a basal cell number was established for further investigations in other SFB 852 trials. The gained expertise on the histochemical characterisation was used in a publication (Kröger *et al*, 2015), where an age related increase in mast cell numbers was noticed.

Moeser et al. (2007) found mast cells to be of great functional importance in gastrointestinal dysfunction induced by early weaning (18 days). They detected increased tryptase levels and enhanced mast cell degranulation which led to a reduction in transepithelial resistance and an increase in mucosal permeability. This mast cell mediated mucosal dysfunction could be prevented by delayed weaning (28 days) (Moeser *et al*, 2007). A cell population involved in mast cell degranulation are the already discussed $CD25^+$ regulatory T cells. They were found to interact tightly with mast cells within their microenvironment, resulting in inhibition of the mast cell degranulation response (Frossi *et al*, 2011). Therefore another point of interest in this thesis was to study interactions between mast cells and $CD25^+$ regulatory T cells in the mucosal network. Since degranulated mast cells - so called "phantom mast cells" - are difficult

to detect via histochemistry (Kaliner & Metcalfe, 1992), immunohistochemistry was used in the studies of Rieger (data not published) to further characterize mucosal mast cells. The immunohistochemical phenotypic characterisation of mast cells in the samples was not reliably possible. Although a broad panel of antibodies was tested, a suitable antibody for embedded specimens was not found, preventing further analyses.

7.3.2. Comparability of results

The problem of tissue shrinkage, addressed in the study of Rieger et al. (2013) (chapter 6) proved to be an important factor with a fundamental impact when comparing samples processed with different fixatives. It was concluded that quantification results have to be adjusted accordingly (e.g. via a shrinkage factor). Otherwise they are not comparable.

An example for the importance of comparison of histology with other methods is the study of Kreuzer et al. (2014) - already commented on. It was shown that differences could be observed only in magnetic sorted lymphocytes, not in whole tissue samples and thus, it was important to examine proposed immune effects in purified immune cells, to be able to find these effects.

And last but not least, also in the studies by Rieger et al. (2015b and 2013) (chapter 5 and 6) the heterogeneous outcome of different immunohistochemical and histochemical staining protocols, resulting in a great variation in the amount of detected *Salmonella* as well as mast cells, shows the challenge to achieve comparability of different experiments.

These examples show the importance to normalize date if they should be compared with one another, the importance to use complementing methods to verify them and the potential which lies in the improvement of already existing protocols.

8. Summary / Zusammenfassung

8.1. Summary of the PhD-Thesis

Title of the PhD thesis: The Intestinal Mucosal Network in the Pig:
A Histological View on Nutrition-Microbiota-Pathogen-Host-Interactions

This thesis was designed under the umbrella of the collaborative research centre (German: Sonderforschungsbereich) “SFB 852 Nutrition and intestinal microbiota - host interactions in the pig”, where different feeding strategies for pigs were examined *in vivo*, including probiotic treatment. There are many literature reports on the efficacy of the frequently-used probiotic *Enterococcus faecium (E. faecium)*. The described effects range from no effect at all to a positive influence on performance (e.g. weight gain or feed conversion ratio) or protection from pathogens or reduced resistance to pathogens. Since the intestine is the interface where nutrition, microbiota, pathogens and host meet, the objective of the thesis was to further our knowledge on the effect of *E. faecium* in a *Salmonella* challenge situation, with particular focus on critically involved components of the intestinal mucosal network.

Chapter 1 gives a general introduction on the pig as one of the most important farm animals for meat production as well as for biomedical research. For a long time, antibiotic growth promotors (in-feed-antibiotics) were used to compensate for the negative effects of intensive pig husbandry. It is known that the overly intensive and uncontrolled use of antibiotics affects human as well as animal health due to a rise in multiple resistances of bacterial infections. Consequently, research efforts of the SFB were focused on feed additives, particularly on mechanistic studies on the effects of probiotics.

The field of work for this thesis was the “Histology Platform”, whose purpose was to apply and refine a broad spectrum of histological methodologies to the porcine organism in the course of several feeding trials. The effects of different nutritional strategies on gastrointestinal histological parameters under either normal housing conditions or in challenge situations were to be examined.

Chapter 2 gives a literature overview on the pig as a model organism in biomedical sciences; porcine intestinal morphology, physiology and immunology; the intestinal mucosal network; nutritional influences on microbiota, pathogens and host and histological techniques applied to investigate the gastrointestinal tract.

The objective of this thesis as well as the hypotheses are summarized in **chapter 3** and are also found in the following descriptions.

Chapters 4, 5 and **6** cover the three main publications for this thesis.

Chapter 4 reports on intraepithelial lymphocyte numbers and histomorphological parameters in the porcine gut after *Enterococcus faecium* NCIMB 10415 feeding in a *Salmonella* Typhimurium challenge. Morphological parameters and the number of intraepithelial lymphocytes (IEL) were evaluated for the effect of the factors "time post infection/age" and "probiotic treatment" by light microscopy. The time post-infection had significant effects ($P < 0.05$) on the treated animals as well as in the control animals. Older animals showed longer and wider villi, deeper and wider crypts, a higher villus enlargement factor, a higher ratio between villus and crypt enlargement factors as well as more intraepithelial lymphocytes. Probiotic treatment resulted in a non-significant tendency for longer villi ($P = 0.037$), a slightly, but non-significant, higher ratio of villus surface/crypt circumference enlargement factors ($P = 0.046$) and significantly more IEL ($P < 0.025$). As the most important result, the population of intraepithelial lymphocytes situated at the nuclear level of the epithelium was identified to be strongly influenced by probiotic treatment ($P = 0.004$ at villus tip and $P < 0.001$ at villus base). It was concluded that the probiotic may have an immune modulatory effect by increasing the number of intraepithelial lymphocytes. These results confirmed the hypothesis that favourable effects of *E. faecium* treatment under a *Salmonella* challenge would involve beneficial changes in performance and immunological related parameters of the intestinal mucosal network. As an indicator for enhanced performance, the mucosal surface available for nutrient absorption was slightly enlarged (1.14-1.31-fold increase in absorptive surface related parameters "villus length", "enlargement factor villi" and "ratio of villus surface/crypt circumference enlargement factors"). The epithelial barrier defending or regenerating intraepithelial lymphocytes were increased (1.09-2.32-fold), wich is a sign for improved immune protection. As an additional indicator for improved immune protection, the number of immunohistochemically detectable bacteria (*Salmonella*) invading the mucosa was expected to be significantly lower. Due to technical issues (see chapter 5), this hypothesis could not be tested.

Chapter 5 reports on the enhancement of immunohistochemical detection of *Salmonella* in tissues of experimentally infected pigs. Samples were obtained from a challenge trial in which piglets were infected with *Salmonella enterica* serovar Typhimurium DT104. Tissue samples were fixed in Zamboni's fixative and paraffin-embedded. Different immunohistochemical staining protocols were evaluated. *Salmonella* was detected in varying amounts in the tissues, and detergents like Triton X-100 or Saponin were found to enhance the sensitivity of the detection method. Additionally, a detection limit for *Salmonella* in immunohistochemical preparations was estimated (10^2-10^3 CFU per g tissue). It was concluded that the use of detergents could result in a higher sensitivity in the immunohistochemical detection of salmonellae. To the best of my knowledge, this is the first

report on this issue. The results confirmed the hypothesis that (species-) specific histological protocols improve the detection of pathogens within porcine tissues.

Since mast cells were identified as a central cell population with a multitude of physiological and pathological functions, including regulation of intestinal barrier function and host defence within the intestinal mucosal network, it was hypothesised that these cells are influenced by probiotic and other treatments in the SFB 852 trials. A species-specific protocol was to be established to reliably quantify mast cells in porcine tissues and find a basal cell number for further investigations in other SFB 852 trials. This aim was accomplished and in **Chapter 6** the evaluation of different fixatives for histochemical staining techniques for porcine intestinal mast cells is described, including the effect of tissue shrinkage during fixation and embedding. Different tissue fixation and staining methods were evaluated in the porcine intestine. Metachromatic staining of mast cells was found to be critically dependent on the fixation and staining technique. The study revealed that zinc salt fixation preserved metachromatic staining in mast cells, which is the first report on this topic. Polychromatic methylene blue was deemed the optimal staining and in order to compare mast cell counting results between different fixation methods, tissue shrinkage has to be taken into account. These results also confirmed the hypothesis that (species-) specific histological protocols improve the detection and identification of immune cells within porcine tissues.

In a general discussion (**chapter 7**), 3 topics concerning the intestinal mucosal network are discussed that turned out to be important issues in histological studies on nutrition-microbiota-pathogen-host-interactions: developmental changes, nutrition-related changes and methodological aspects.

8.2. Zusammenfassung der Dissertation

Dissertation zum Thema: Das schleimhautassoziierte Netzwerk im Darm des Schweins: Eine histologische Untersuchung von Ernährung-Mikrobiota-Pathogen-Wirts-Interaktionen

Die vorliegende Doktorarbeit wurde im Rahmen des Sonderforschungsbereiches „SFB 852“ konzipiert, in dem verschiedene Fütterungskonzepte, einschließlich Probiotikagabe, für Schweine *in vivo* untersucht wurden. In der Literatur finden sich verschiedene Berichte über die Effektivität des Probiotikums *Enterococcus faecium (E. faecium).* Sie reichen von gar keinem Effekt, über eine positive Wirkung auf die Leistung (z.B. Gewichtzunahme oder Futterumsatzrate) und einem Schutz vor Pathogenen, bis hin zu einer reduzierten Resistenz gegenüber Pathogenen. Da der Darm die Schnittstelle ist an der Ernährung, Mikrobiota, Krankheitserreger und Wirt aufeinandertreffen, war das Ziel der Arbeit die Vermehrung unseres Wissens über die Wirkung von *E. faecium* in einem Salmonellen-Infektionsversuch. Besonderer Fokus lag dabei auf beteiligten Komponenten mit kritischen Funktionen innerhalb des schleimhautassoziierten Netzwerkes im Darm.

Kapitel 1 gibt einen Überblick über das Schwein, eines der wichtigsten Nutztiere in der Fleischproduktion sowie in der biomedizinischen Forschung. Für eine lange Zeit wurden Fütterungsantibiotika in der intensiven Nutztierhaltung eingesetzt, um negative Effekte auszugleichen, die dieses Haltungssystem mit sich bringt. Es ist bekannt, dass ein übermäßig intensiver und unkontrollierter Einsatz von Antibiotika die Human-, wie auch die Tiergesundheit beeinflusst und zu einem Anstieg von Infektionen mit multiresistenten Keimen führt. Die Forschungstätigkeit des SFB 852 war daher auf Fütterungszusätze ausgerichtet, im speziellen auf mechanistische Studien zum Effekt von Probiotika.

Das Arbeitsfeld dieser Dissertation war die „Histologie Plattform“, deren Aufgabe darin bestand in Fütterungsversuchen mit Schweinen ein breites Spektrum an histologischen Methoden am porzinen Organismus anzuwenden und zu verbessern. Der Effekt verschiedener Fütterungsstrategien auf histologische Parameter sollte unter normalen Haltungsbedingungen und auch in Infektionsversuchen untersucht werden.

Kapitel 2 gibt einen Einblick in die Literatur über das Schwein als Modellorganismus in der biomedizinischen Forschung; porzine Darmmorphologie, -physiologie und -immunologie; das schleimhautassoziierte Netzwerk im Darm; ernährungsbedingte Einflüsse auf Mikrobiota, Pathogene und den Wirt sowie histologische Techniken, die zur Untersuchung des Magendarmtraktes eingesetzt werden können.

Das Ziel und die Hypothesen dieser Arbeit werden in **Kapitel 3** zusammengefasst. Sie sind ebenfalls in der folgenden Beschreibung enthalten.

Kapitel 4, 5 und **6** enthalten die drei Hauptpublikationen für diese Arbeit.

Kapitel 4 berichtet über intraepitheliale Lymphozyten-Zahlen und histomorphologische Parameter im Schweinedarm nach Fütterung von *Enterococcus faecium* NCIMB 10415 innerhalb eines Infektionsversuchs mit *Salmonella* Typhimurium. Lichtmikroskopisch wurde die Wirkung der Faktoren "Zeit nach der Infektion / Alter" und "probiotische Behandlung" auf morphologische Parameter und die Anzahl der intraepithelialen Lymphozyten (IEL) untersucht. Die vergangene Zeit seit der Infektion zeigte signifikante Auswirkungen ($P < 0.05$), sowohl in der Behandlungs- als auch in der Kontrollgruppe. Ältere Tiere hatten längere und breitere Zotten, tiefere und breitere Krypten, einen höheren Zotten-Vergrößerungsfaktor, ein höheres Verhältnis zwischen Zotten- und Krypten-Vergrößerungsfaktoren sowie mehr intraepitheliale Lymphozyten. Die probiotische Behandlung führte tendenziell, aber nicht signifikant, zu längeren Zotten ($P = 0.037$) sowie einem leicht erhöhten Verhältnis von Zotten-Oberfläche/Krypten-Umfang-Vergrößerungsfaktoren ($P = 0.046$) und signifikant mehr intraepithelialen Lymphozyten ($P < 0.025$). Als wichtigstes Ergebnis wurde festgestellt, dass die auf Kernebene des Epithels gelegene Population der intraepithelialen Lymphozyten stark durch die probiotische Behandlung beeinflusst war ($P = 0.004$ an der Zottenspitze und $P < 0.001$ an der Zottenbasis). Daraus wurde geschlossen, dass das Probiotikum durch Erhöhung der Anzahl der intraepithelialen Lymphozyten eine immun-modulierende Wirkung haben könnte. Diese Ergebnisse bestätigen die Hypothese, dass positive Auswirkungen einer *E. faecium* Behandlung während einer Salmonellen-Infektion mit positiven Veränderungen in den Leistungs- und immunologischen Parametern des schleimhautassoziierten Netzwerkes verbunden sein müssten. Als Indikator für eine verbesserte Leistung war die für die Nährstoffaufnahme zur Verfügung stehende Schleimhautoberfläche leicht vergrößert (1.14-1.31-fache Zunahme der die absorptive Oberfläche betreffenden Parameter “Zottenlänge”, “Vergrößerungsfaktor Zotten” und “Verhältnis der Vergrößerungsfaktoren Zottenoberfläche/Kryptenumfang”). Die intraepithelialen Lymphozyten zur Verteidigung oder Regeneration der epithelialen Barriere waren vermehrt (1.09-2.32-fach). Dies ist ein Hinweis auf einen verbesserten Immunschutz. Als zusätzlicher Indikator für einen verbesserten Immunschutz sollte die Zahl der immunohistochemisch nachweisbaren Bakterien (*Salmonella*), die in die Schleimhaut eindringen, signifikant niedriger sein. Aufgrund technischer Aspekte (siehe Kapitel 5), konnte diese Hypothese nicht getestet werden.

Kapitel 5 berichtet über die Verbesserung des immunhistochemischen Nachweises von *Salmonella* in Geweben experimentell infizierter Schweine. Zur Untersuchung kamen Proben eines Infektionsversuches, in denen Ferkel mit *Salmonella enterica* Serovar Typhimurium DT104 infiziert waren. Gewebeproben wurden in Zambonis Fixiermittel fixiert und in Paraffin eingebettet. Anschließend erfolgte die Erprobung und Auswertung verschiedener immunhistochemischer Färbeprotokolle. *Salmonella* konnte in unterschiedlichen Mengen in den Geweben nachgewiesen werden und Detergentien, wie Triton X-100 oder Saponin,

erhöhten die Empfindlichkeit des Nachweisverfahrens. Zusätzlich wurde eine Nachweisgrenze für *Salmonella* in immunohistochemischen Verfahren geschätzt (10^2-10^3 CFU pro g Gewebe). Daraus ergab sich die Schlussfolgerung, dass die Verwendung von Detergentien zu einer höheren Empfindlichkeit im immunhistochemischen Nachweis von Salmonellen führen kann. Nach bestem Wissen ist dies die erste Abhandlung zu diesem Thema. Die Ergebnisse bestätigten die Hypothese, dass (Spezies-) spezifische histologische Protokolle notwendig sind, um die Erkennung von Krankheitserregern in Schweinegewebe zu verbessern.

Da Mastzellen eine zentrale Zellpopulation mit einer Vielzahl von physiologischen und pathologischen Funktionen darstellen, einschließlich der Regulierung der Darmbarrierefunktion und Wirtsverteidigung im schleimhautassoziierten Netzwerk, wurde die Hypothese aufgestellt, dass diese Zellen durch probiotische und anderen Behandlungen in den SFB 852 Studien beeinflusst sein müssten. Ein Spezies-spezifisches Protokoll sollte etabliert werden, um Mastzellen in Schweinegeweben zuverlässig zu identifizieren und quantifizieren und eine basale Zellzahl für weitere Untersuchungen in anderen SFB 852 Studien zu finden. Dieses Ziel wurde erreicht und somit wird in **Kapitel 6** über die Evaluierung verschiedener Fixiermittel für histochemische Färbetechniken von Mastzellen im Schweinedarm berichtet. Besondere Berücksichtigung findet dabei die Gewebeschrumpfung, die während der Fixierung und Einbettung auftritt. Verschiedene Gewebefixierungs- und Färbemethoden wurden am Schweinedarm untersucht. Die metachromatische Färbung von Mastzellen erwies sich als kritisch abhängig von der Fixierungs- und Färbungstechnik. Desweiteren ergab die Studie, dass eine Zinksalz-Fixierung die metachromatische Färbung von Mastzellen erhält. Dies ist der erste Bericht zu diesem Thema. Polychromatisches Methylenblau hat sich als optimale Färbung zum Mastzellnachweis herauskristallisiert. Um Mastzellen-Zählergebnisse zwischen den verschiedenen Fixierungsmethoden zu vergleichen, musste die Gewebeschrumpfung berücksichtigt werden. Diese Ergebnisse bestätigten auch die Hypothese, dass (Spezies-) spezifische histologische Protokolle die Detektion und Identifizierung von Immunzellen in porzinen Geweben verbessern.

In einer allgemeinen Diskussion (**Kapitel 7**) werden 3 Themen aufgegriffen, die das schleimhautassoziierte Netzwerk im Darm betreffen. Diese haben sich als wichtig in den histologischen Studien zu Ernährungs-Mikrobiota-Pathogen-Wirt-Interaktionen erwiesen: entwicklungsbedingte Veränderungen, ernährungsbedingte Veränderungen und methodische Aspekte.

9. References

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10. List of publications

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12. Declaration of academic honesty

I hereby confirm that the text at hand is solely my own work. I assure that I only used the cited sources and the thesis has not been submitted in any form for another degree at any university or other institute.

Berlin, 15.06.2016

Juliane Rieger