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Molecular and functional analysis of the porcine and murine small intestinal tight junction

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List of abbreviations

FAE	Follicle-associated epithelium
Fig.	Figure
FITC	Fluorescein isothiocyanate
IgA	Immunglobulin A
IgG	Immunglobulin G
INF-γ	Interferon-γ
LF	Lactoferrin
MDCK II	Madin Darby Canine Kidney
p.	Page
PP	Peyer's patch
R^{epi}	Epithelial electrical resistance
R^{sub}	Subepithelial electrical resistance
TGF-β	Transforming growth factor-β
TER	Transepithelial resistance
TNF-α	Tumor necrosis factor-α
TJ	Tight junction
VE	Villous epithelium

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Chapter 1: Introduction

Epithelial cell layers cover the inner and outer surfaces of a body, forming a barrier between the environment and the organism (Powell, 1981). The most relevant structure for the epithelial barrier integrity is the tight junction (TJ). The TJ is the most apical lateral cell connection between epithelial cells and is organized in strands (Farquhar & Palade, 1963; Staehelin, 1973). Its main functions are the control of paracellular flux (gate function) and the inhibition of intramembranous diffusion of proteins (fence function). Different membrane spanning proteins serve as the backbone of the TJ strands which bridge a gap between neighboring cells (Staehelin, 1973).The largest family of TJ proteins is the family of claudins. Currently 27 members are known (Mineta *et al.,* 2011), and many of them have been linked to specific functions (Markov *et al*., 2015). Expression and localization of claudins in TJ complexes determine the permeability of the specific tissue, enabling a classification into leaky and tight epithelia (Amasheh *et al.,* 2009a; Anderson & Van Itallie, 2009). The ability to control the permeability enables the claudins to be classified as sealing or pore-forming (Amasheh *et al*., 2002). Furthermore, TJ are generally classified as permselective, since they express specific preferences for different ions, charges and molecular sizes (Powell, 1981).
Functional analysis of epithelial cell layers can be carried out by employing the Ussing chamber technique (Ussing & Zerahn, 1951). This electrophysiological method allows measurement of active transport across epithelial monolayers. Simultaneously, paracellular flux can be measured by using specific marker molecules. Subsequent calculations of the apparent permeability allow direct comparison of paracellular transport rates across different tissues.
TJ protein expression in the intestinal epithelium has been characterized in detail recently (Markov *et al.,* 2010). It was demonstrated that claudin expression levels are strongly associated with the epithelial barrier function. While pore-forming claudins show a stronger expression in the proximal, leaky segments of the intestine, sealing claudins are most abundantly expressed in the distal part (Markov *et al*., 2010). These results can be linked to the physiological function of the different gut segments. A leaky barrier is in accordance with the resorptive function of the small intestine, whereas a tight epithelial barrier in the distal part is a requirement for hormonal regulation of fluid homeostasis (Amasheh *et al.*, 2009b; Hanukoglu & Hanukoglu, 2016).

This thesis focused on two different aspects of the epithelial barrier function:

1. **Effects of milk on the intestinal epithelial barrier function**
   The supply of milk is essential for the survival of newborn mammals because milk is the sole source of nutrition. Maximal absorption of nutrients would be of advantage to ensure full energy supply in this developmental phase. Therefore, this thesis focused on the effects of milk on epithelial barrier function and tight junction protein expression in a porcine model.

2. Barrier properties in Peyer's patches (PP)

PP are a part of the gastrointestinal immune system and are located directly underneath the epithelial layer. They serve as the first line of defense, when challenged by an antigen. PP are covered by a specialized epithelium, which regulates the interaction between antigen and follicle. The functional and molecular characterization of the specialized epithelium is reported in this thesis with a specific focus on TJ protein expression in murine and porcine tissue.

Chapter 2: Literature review

2.1. The epithelial barrier

The epithelial barrier is a physiological necessity for tissue and organ functions, as it allows compartmentalization in the body (Marchiando *et al.*, 2010). Furthermore, the epithelial barrier protects the organism from undesirable physical or chemical harm as it prevents putative pathogenous microorganisms or toxic substances from entering the body (Pohl *et al.*, 2009). Moreover, a tight epithelial barrier protects from dehydration (Furuse *et al.*, 2002) and mediates controlled and regulated absorption and secretion (Amasheh *et al.,* 2009b; Markov *et al*., 2017).
Epithelial cells connect to the underlying tissue, the basement membrane, *via* their basal side. The apical side is oriented towards the lumen of a hollow organ or towards the outer surface. This strict separation between the apical and basal side of the cell is a special characteristic of epithelial cells. It is a requirement to uphold the concentration gradient between the intracellular and extracellular levels of different electrolytes, which then enables an oriented uptake of nutrients and other substances. Furthermore, epithelial cells are closely connected *via* an abundance of cellular junctions (Hees, 1990).

A number of different cellular junctions can be detected in multicellular organisms. The schematics of epithelial cell junctions are illustrated in **Fig. 1**. Classified by function and morphology these cellular junctions can be separated into four groups:

1. **Tight junctions** (TJ) are found in mammalian epithelial cell layers (Furuse *et al*., 2002). They serve as a regulator of the paracellular barrier, connect neighboring cells and enable an oriented uptake of substances (see chapter 2.2).
2. Proteins of **anchoring junctions** extend through cells, connect neighboring cytoskeletal elements and reach into the extracellular matrix facilitating the development and physiology of epithelial tissue (Harris, 2012). Research to date has identified three types of anchoring junctions: 1) desmosomes, 2) hemidesmosomes and 3) adherens junctions. The differentiation was based on specific cytoskeletal anchors and linking proteins.
3. **Communicating junctions**, also known as gap junctions, are formed to establish a direct intercellular connection between neighboring cells and permit easy exchange of cytoplasm. The pore is made up of six connexin proteins, forming a cylinder with a central pore (Evans *et al.*, 2002). Gap junctions are vital for mammalians, as they enable proper signal transduction in cardiomyocytes (Weidmann, 1966).
4. The geometric features of **tricellular junctions** require specific organization. The TJ protein tricellulin is heavily involved in the regulation of the tricellular junction permeability, accounting for about 9 - 32 % of epithelial conductance (Krug, 2017). Furthermore, tricellular junctions are also required in the process of cellular division, as the connection to the spindle apparatus serves as the pulling force during mitosis (Bosveld *et al*., 2016).

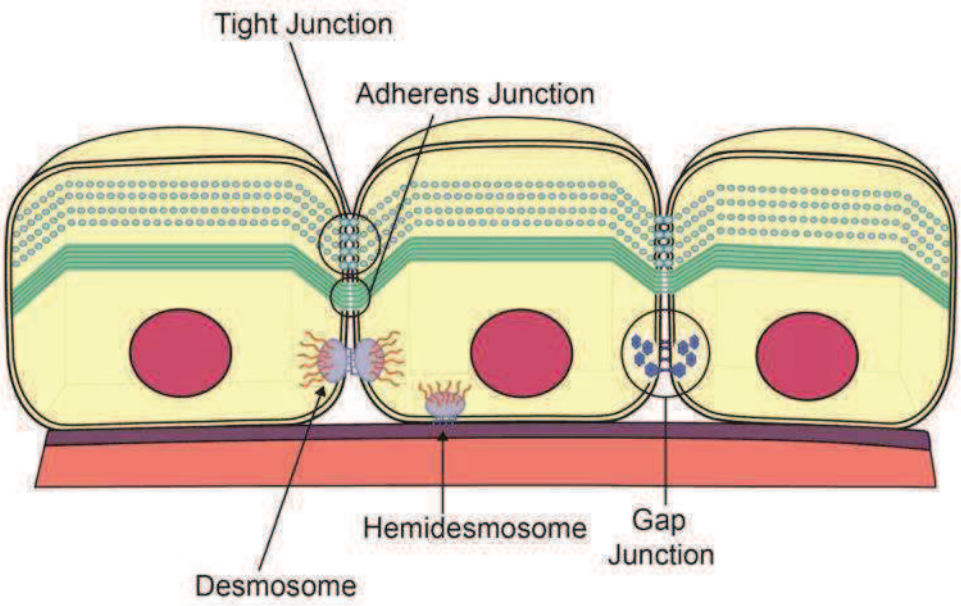

Figure 1: The schematics of epithelial cell junctions. Tight junctions surround the upper part of the cell, form a paracellular barrier and therefore regulate the paracellular transport. Anchoring junctions are represented by adherens junctions, hemidesmosomes and desmosomes, which either connect cells directly or to the underlying membrane. Gap junctions form a connection between the intracellular cytoplasm of two neighboring cells allowing direct communication and faster transmission of electrical impulses (Levendoski *et al*., 2014).

As this thesis is focused on the characterization of the intestinal epithelial barrier function, the special features of the intestinal epithelium had to be regarded.

The overall small intestinal mucosa is organized in villi and crypts. Both enhance the intestinal surface area for increased nutrient resorption. The main cell type in the intestinal epihelium is the enterocyte. The small intestinal enterocyte is of cylindrical form and contains oval nuclei, which are located in parallel with the basal membrane. Most cells carry surface variations such as the microvilli, which form the brush border (Mooseker, 1985). The brush border enhances the epithelial surface and is where different digestion enzymes are located (Hees, 1990). To protect the epithelium against the digestion enzymes, the microvilli are covered by a carbohydrate-enriched coating known as the glycocalyx (Butler *et al.,* 1983). The enterocytes are laterally closely connected via TJ proteins. On the apical side the *zonulae occludentes* prevents uncontrolled entry of substances, while the intracellular gap on the basolateral side remains open (Hees, 1990).

Since the porcine and murine small intestinal TJ was the main subject of this thesis, the importance, function and structure will be highlighted in the following chapters.

2.2. The structure and function of the tight junction

TJ are formed when membranes of two adjacent epithelial cells come into contact with each other. Through the interaction of TJ proteins, an intercellular barrier is formed. Conjoined, the *zonula adhaerens*, *macula adhaerens* and the TJ *(zonula occludens)* form the junctional complex which connects epithelial cells and facilitates the function of organs or tissues (Farquhar & Palade, 1963). Closely located at the apical part of the epithelial cells, the TJ surrounds the whole cell and connects it to neighboring cells through intramembranous proteins. This connection is tissue specific and is used to regulate the paracellular pathway (Markov *et al*., 2010).

TJ are an important part of the cellular structure as they form a needed framework which serves as the backbone of epithelial tissues. Their most important functions are:

1. **The mechanical connection of neighboring cells** (Irie *et al*., 2004).
2. **The gate function:** The TJ strictly controls the paracellular flux and therefore the uptake of ions and smaller molecules. This serves as a precise control over what kind of substances or potentially harmful agents are able to enter the body (Anderson *et al*., 2004).
3. **The fence function:** Located in the upper part of the cellular membrane, the TJ serves as a diffusion inhibitor for other intramembranous proteins. This function maintains the polarity of the epithelial cell layer. This is important for the directional uptake of specific nutrients, such as amino acids and glucose (Gumbiner, 1987).

Visualization of the TJ was first successfully performed in 1963. The interactions between membranes of neighboring cells in numerous spots were identified as “kissing points” (Farquhar & Palade, 1963). Later research used electron microscopy to reveal multiple connecting strands of transmembrane particles between adjacent cells. These form the physical barrier that closes the paracellular gap (Staehelin, 1973). The strands are all part of transmembrane proteins, which are located within the cellular membranes. While extracellular domains interact to form barrier regulating strands, intracellular domains connect the TJ to parts of the cytoskeleton (Hartsock & Nelson, 2009).
The classification of epithelia as leaky or tight is dependent on TJ protein expression and formation (Anderson & Van Itallie, 2009). The tightness of an epithelial layer can be expressed with the electrophysiological value transepithelial resistance (TER), which can be measured by the Ussing chamber technique (see chapter 2.2.2.).
First experiments led to the conclusion that the number of extracellular TJ strands was positively correlated to the tightness of the epithelia (Claude & Goodenough, 1973). Later, Claude discovered that the relationship between the number of TJ strands and the tightness of the barrier is logarithmic (Claude, 1978). There is also a dynamic regulatory effect involved as certain strands contain pores which can be opened or closed (Sasaki *et al*., 2003).

Research to date concludes that the number of strands does not determine the tightness of the barrier. Instead, it is determined by expression levels of TJ proteins.
A tissue with tight TJ such as the skin is able to withstand the force of higher electrochemical gradients and uphold the concentration differences through blocked paracellular flow. By contrast leaky TJ are formed in tissues such as the small intestine where large amounts of isosmotic fluids must be transported. TJ are classified as permselective because a preference for different ions, charges or molecular sizes can often be observed (Powell, 1981).
Research to date has shown that TJ have two components that are responsible for paracellular transport (**Fig. 2**):

1. **Pores:** The pathway through the pores has a high capacity that consists of size- and charge-selective small pores. These allow the passage for solutes that are smaller than 4 Å in radius. This pathway is presumed to be formed by interactions of the TJ protein family of claudins. (Knipp *et al*., 1997; Watson *et al*., 2001; Van Itallie *et al*., 2008). Single claudins have been characterized as ion and water-selective channels (Amasheh *et al*., 2002; Amasheh *et al*., 2011).
2. **Leak:** The pathway through leaks has a low capacity for bigger solutes, which is made up of larger discontinuities in the barrier. This pathway is neither charge- nor size-selective and depends on the cell-cell adhesion and cytoskeleton (Bruewer *et al*., 2004; Ivanov *et al*., 2005).

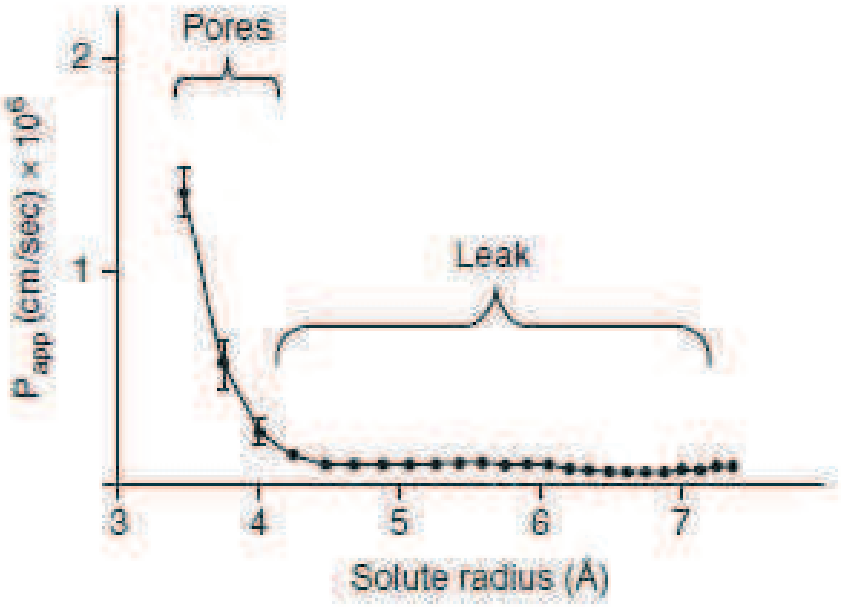

Figure 2: Simplified scheme of two pathways of tight junctions. The pores form the pathway with higher transport capacity. They are size- and charge-selective and are regulated by claudins. The leak pathway is neither size- nor charge-selective and is

dependent on the cytoskeleton. It is made up of barrier discontinuities (Anderson *et al*., 2009).

2.2.1. The significance of intestinal claudins and other tight junction proteins

Visualization of tight junction strands revealed a branching and anastomosing network between neighboring cells (Claude & Goodenough, 1973). The TJ strands form tight connections that heavily regulate the intercellular transport (Staehelin, 1973). Within these strands, a number of tetraspan TJ proteins, including occludin, tricellulin and the family of claudins have been identified to contribute to specific gate properties of epithelia (Furuse *et al*., 1993; Furuse *et al*., 1998; Ikenouchi *et al*., 2005). Now classified as a marker for TJ protein localization (Amasheh *et al*., 2002), occludin was first discovered in 1993 (Furuse *et al*., 1993). Although it was the first molecule that could be directly linked to the TJ, the functional contributions of occludin are still being researched (Schulzke *et al*., 2005). Nevertheless the search for further molecular components of the TJ began, and only five years later the first claudins were identified (Furuse *et al*., 1998). The functional characterization of single claudins resulted in the classification of sealing, pore-forming and ambiguous claudins. This classification allowed a strong correlation of specific expression levels and localization with barrier properties (Amasheh *et al*., 2011). Combinations of claudins within TJ strands on a molecular level have been reported indicating specific interactions in defined clusters (Van Itallie *et al*., 2014; Markov *et al*., 2015).

Focusing on the intestine, the epithelium shows segment-specific expression levels of TJ proteins (**Fig. 3**), which has been demonstrated to be in accordance with barrier function of rat duodenum, jejunum, ileum and colon (Markov *et al*., 2010). A number of claudins are found in mammalian intestine, including claudin-1, -2, -3, -4, -5, -7, -8 and -12 as shown in rat (Markov *et al*., 2010; Rahner *et al*., 2001), mouse (Holmes *et al*., 2006; Fujita *et al*., 2006), human (Bürgel *et al*., 2002; Zeissig *et al*., 2007) as well as porcine intestinal epithelial cells (Schierack *et al*., 2006; Zakrzewski *et al*., 2013).

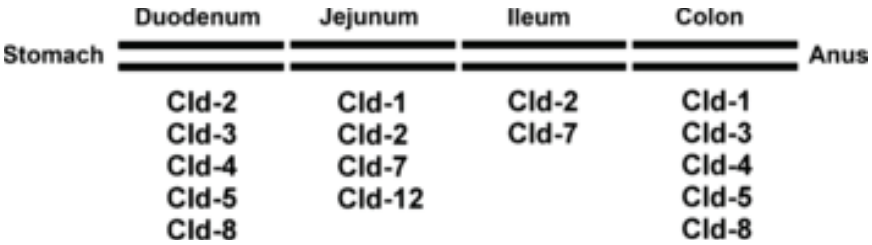

Figure 3: Expression of major claudins in intestinal segments reflects local barrier properties. Small intestinal segments, such as the duodenum, jejunum and ileum show stronger expression of claudin-2, -7 and -12, whereas in the colon, the strongest expression of sealing claudin-1, -3, -4, -5 and -8 was reported (Amasheh *et al*., 2011). This is in accordance with the permeability of these intestinal sections.

A main parameter of TJ integrity is the epithelial barrier function, which can be measured and distinguished from submucosal tissue layers (R^{sub}) as epithelial resistance (R^{epi}) by impedance spectroscopy (Gitter *et al*., 1998). In proximal segments of rat intestine R^{epi} was reported to decrease along the proximal distal axis from the duodenum to the ileum, followed by a strong increase in R^{epi} in the colon (Markov *et al*., 2010). Barrier properties were in accordance with the localization of claudins. The colon showed the highest expression of tightening claudin-1, -3, -4, and -8 in the surface epithelium, followed by the duodenum, which also showed a strong expression of claudin-2. The jejunum and the ileum were characterized by a lower expression of tightening TJ proteins and an even higher expression of paracellular permeability mediators (Markov *et al*., 2010).
In a functional context, the strongest deviation from a gradual tightening along the proximal-distal axis occurs in the duodenum. However, the relatively high R^{epi} in this segment and higher expression of tightening TJ proteins is in accordance with important physiological implications, as the epithelium faces rapid luminal changes of osmolarity, bile salts and pH (Markov *et al*., 2010).
Localization of claudins along the crypt-to-villus axis of intestinal tissues also gives indication of the functional contribution of claudins. The majority of claudins are expressed in the surface epithelium representing a primary role for barrier properties, even though single claudins vary in localization. A significant example is claudin-2, which is localized in colon crypts, but not in the surface epithelium in healthy colon (Rahner *et al*., 2001). Moreover claudin-1, -3, -5 and -7 can also be detected in subjunctional membrane compartments (Holmes *et al*., 2006). This localization may represent a storage pool but may also be involved in cell signaling and adhesion (Li *et al*., 2004).

The role of claudins in intestinal pathophysiology has been demonstrated by reports of an increased claudin-2 expression in colonic mucosa of patients with inflammatory bowel diseases. In ulcerative colitis, pouchitis, and Crohn's disease, claudin-2 is upregulated (Amasheh *et al*., 2009c; Zeissig *et al*., 2007; Heller *et al*., 2005), and paralleled by a decrease of tightening claudins, which causes a general dysfunction of the epithelial barrier. Moreover, many members of the claudin family have been found to be prognostic and are diagnostic tumor markers (Gröne *et al*., 2007; Agarwal *et al*., 2009).

Since the expression of claudin proteins correlates with the intestinal barrier function and their properties, this coherence will be highlighted in the following chapter of this doctoral thesis.

2.2.2. Barriology

The recent discovery of TJ proteins, namely the family of claudins (Furuse *et al*., 1998) and occludin (Furuse *et al*., 1993) drew more interest to this newly discovered field of biology. The intestinal epithelium faces the challenging task of uptaking nutrients and protecting against potentially dangerous pathogens at the same time. An impairment of the intercellular

connections could lead to short-term, but also chronic and fatal diseases. Therefore the detailed knowledge of the structure, function and regulation of responsible TJ proteins is of general importance.

Functional analyses of epithelial barrier properties are typically carried out with electrophysiological methods and paracellular flux measurements, which allows for subsequent calculation of permeability.
In 1951, Hans Ussing developed an experimental setup (**Fig. 4**) which enabled scientists to measure the barrier and transport properties across epithelial cell layers (Ussing & Zerahn, 1951). Epithelial tissues or cells grown on permeable supports are mounted in a chamber, which is connected to two fluid reservoirs, leading to a strict separation of the mucosa and serosa. Constant circulation of experimental buffer is ensured by the permanent application of carbogen (95 % O_2, 5 % CO_2). *In vitro* conditions are guaranteed by setting the temperature to 37 °C and maintaining a pH of 7.4.
As TJ proteins serve as a diffusion inhibitor within epithelial cell membranes vectorial transport of ions or molecules is possible. Since the sodium potassium pump is located in the basolateral part of the cellular membrane and actively pumps sodium into the extracellular space, the intracellular sodium concentration is kept constant. Due to the concentration gradient, secondary active symporters that are located in the apical membrane transport glucose or amino acids into the epithelial cell. Therefore the sodium potassium pump enables intestinal cells to constantly absorb nutrients and transport charged ions.
Active transport through the epithelial cell layer leads to an electrogenic movement of molecules which results in a potential difference between the mucosal and serosal side of the tissue. Applying a counter current, which clamps the transepithelial potential difference to 0 mV enables the measurement of active transport processes across epithelial membranes (short circuit current, I_{sc} [µA / cm²]).

Subsequent calculation, employing Ohm's law allows determination of TER:

$$\mathrm{TER} = \frac{\mathrm{U}}{\mathrm{I_{sc}}}$$

TER	Transepithelial resistance
U	Transepithelial voltage
I_{sc}	Short circuit current

The epithelial barrier function is typically associated with the electrical resistance across the tissue and is expressed as R [Ω · cm²].

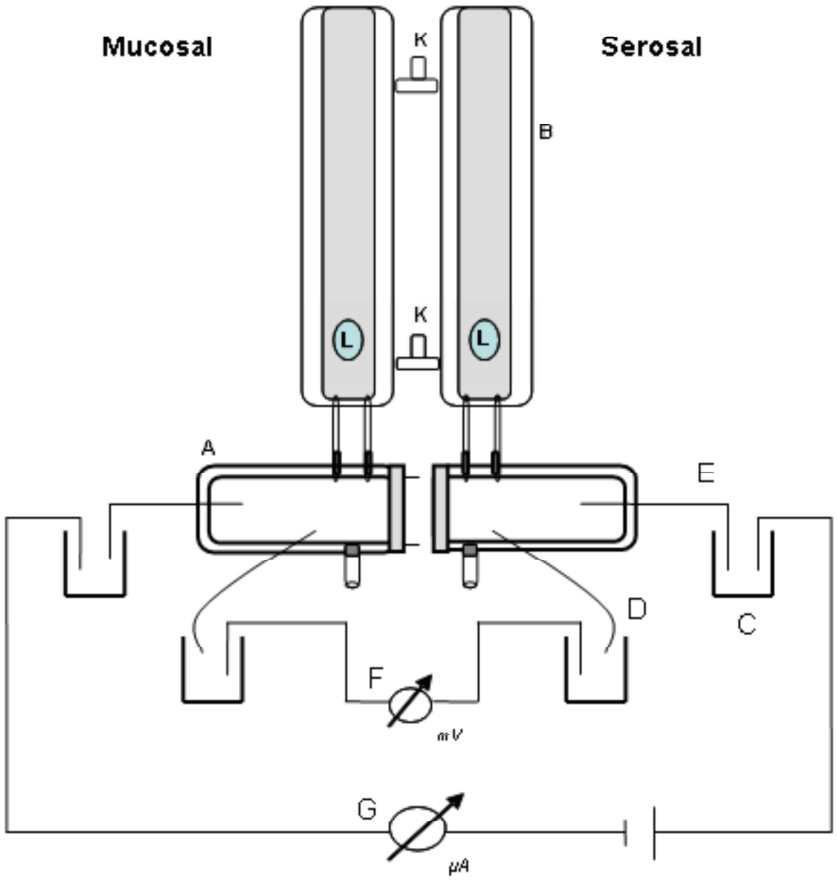

Figure 4: Experimental Ussing chamber setup. Ussing chambers are used for the detection of net ion transport across an epithelium. Ion transport induces a transepithelial electric voltage (U [V]).This voltage can be clamped to 0 when employing a counter current (I [A]). Using Ohm's law, the transepithelial resistance can be calculated. A = Fiberglass chamber, B = Double walled fluid reservoir, C = Electrode holder, D = Potential electrodes, E = Current electrodes, F = Voltmeter, G = Amperemeter, K = Opening for prewarmed waterbath, L = Opening for gas supply (Rabbani, 2010).

Employing an Ussing chamber setup, flux measurements can be carried out. Sodium fluorescein (330 Da) is a widely used marker substance for paracellular permeability. Enlisting paracellular tracer in an experimental setup is a common way to describe the intercellular barrier and explore possible passive transport routes. Therefore sodium fluorescein is added to the apical side of the chamber in a known concentration (c_i). To assess the paracellular flux, buffer samples are taken from the basolateral side of the chamber and photometrically measured for sodium fluorescein. The amount taken is replaced with fresh buffer to ensure there are equal levels of fluid and pressure on both sides of the epithelium.

The transepithelial flux is then calculated as follows (Zakrzewski, 2014):

$$J = \frac{(c_2' - c_1) \cdot V_{chamber}}{\Delta t \cdot A}$$

c_2' denotes the fluorescein concentration in the basolateral compartment, adjusted to the dilution, for sample taking.

$$c_2' = \frac{(C_1 \cdot V_s) + (c_2 \cdot V_{chamber})}{V_{chamber}}$$

J	Flux
c_1, c_2	Concentration at timepoint 1 and 2
c_2'	Corrected c2
Δt	Time in between measurements
$V_{chamber}$	Buffer volume in chambers
V_s	Sample volume
A	Tissue area

Subsequently, the apparent permeability can be calculated as well by taking the initial tracer concentration into account (Zakrzewski, 2014):

$$P = \frac{J}{c_i}$$

P	Permeability
J	Flux
c_i	Initital tracer concentration

2.3. Immunocompetence and nutrition of piglets

This thesis focused on the intestinal barrier function of piglets. Shortly after birth, piglets are already confronted with a plethora of potentially harmful agents. A tight paracellular barrier is essential for controlled interaction of the intestinal immune system, which is represented by Peyer's patches (PP), with antigens.

Morphologically all parts of the porcine immune system are present at birth, functionally however, they are still immature and need several weeks to fully differentiate (Rooke & Bland, 2002). Intestinal T lymphocytes could be detected at birth, but in lower numbers than in older animals (Gaskins, 1998). Moreover, mononuclear cells showed a relatively slow

reaction to antigen contact (Hammerberg *et al*., 1989) and immunoglobulin production was decreased due to a low amount of producing cells in the spleen and bone marrow (Bianchi *et al.,* 1999).

Due to their immature immune system the newborn piglet is devoid of immunoglobulins (Bourne, 1973). There is no exchange of maternal immunoglobulins during gestation, due to the specific structure of the porcine placenta. The so-called *placenta epitheliochorialis* is structured in six successive tissue layers separating the bloodstream of the mother from the fetus's (Schnorr & Kressin, 2006). While the fetus's endothel, mesenchym and chorionepithelia forms one side of the barrier, the other is made up of the mother's *Lamina epithelialis*, *Lamina propria* and the maternal endothelium. Since this barrier does not allow exchange of bigger molecules such as the immunoglobulins, the newborns are highly dependent on their mother's colostrum, which provides nutrients, as well as functional immunoglobulins (Bourne, 1973). As it has been shown that colostrum deprived piglets die within 24 hours after birth (Owen *et al*., 1961), the first hours of life are critical for the survival of the individual.

The absorption of macromolecules is limited to only a few hours after birth, which highlights the importance of this specific timeframe. Important macromolecules for immunity are immunoglobulins. They serve as antibodies against antigen and are produced by the mother after contact. Immunoglobulins G (IgG) facilitate the neutralization of toxins, viruses and bacteria (Schroeder & Cavacini, 2010). Immunogloblins A (IgA) on the other hand are part of the mucosal immune system, because they locally bind antigen and prevent entry (Brandtzaeg, 2013). Immunoglobulins cannot be produced by the offspring directly after birth due to the immature nature of their own immune system, hence the piglets are dependent on their mothers supply (Bourne, 1973). The immunoglobulin levels need to be high enough to protect the piglet until its own immune system has matured and can sufficiently react to antigen contact with the production of specific antigen. It is not surprising that serum IgG levels are positively correlated with the newborn's chance of survival (Hendrix *et al*., 1976). Hence, a successful suckling period for every piglet is of great importance, regarding the number of surviving individuals (Tuchscherer *et al*., 2000). An adequate uptake of colostrum is essential to increase the survival risk for piglets, because IgG serum levels can also be directly linked to the amount of colostrum ingested (Rooke & Bland, 2002). Not only is the immediate risk of death elevated if there is an insufficient amount of colostrum ingested (Drew *et al*., 1988), but also a higher mortality could be shown even after weaning (Varley *et al*., 1986).

A successful passive immunization is dependent on the transfer of immunoglobulins, bioactive peptides, such as growth factors and cytokines and cellular components from the mother's colostrum to the piglet's intestine (Hurley & Theil, 2011). Due to gut closure after 24 to 36 hours after birth (Speer *et al.,* 1957) IgG needs to be resorbed early. IgA on the other hand supports the mucosal immunization by offering local protection (Brandtzaeg, 2013). In accordance, the immunoglobulin concentrations vary during lactation. In colostrum IgG is typically contained in higher concentrations, while there is only a little amount of IgA (Curtis

et al., 1971; Klobasa *et al.,* 1987; Klobasa & Butler, 1987). During later stages of lactation, IgA is contained in larger concentrations. Only one week after birth, IgG secreting cells can be found within the piglet's lymph nodes, bone marrow and spleen, suggesting another form of passive immunization dependent on the supply of colostrum derived cells, namely leukocytes (Tuboly *et al*., 1988; Bianchi *et al.,* 1999). It is important to note that immunoglobulins contained in colostrum represent the current status of immunization of the mothering sow, and therefore only offers specific protection.

The uptake of IgA *via* non-specific pinocytosis has been explained in detail in the 1960s (Payne *et al.,* 1962). This is a special feature of neonatal enterocytes, which enables newborns to absorb intact protein structures and therefore maintain the proteins functionality (Sangild *et al.,* 1997). Moreover, these macromolecules need to be transported further, through the basolateral membrane and into the bloodstream for final metabolism. As the basal transport of IgG is reduced after 24 hours, this timeframe was defined as gut closure (Rooke *et al*., 2002). The most probable initiating factor is the proper intake of nutrients, as fasting piglets delays closure (Lecce *et al*., 1962), and the intake of sugars, as glucose and lactose induce closure (Lecce, 1966; Werhahn *et al*., 1981).

Although a sufficient IgG uptake during the first 24 hours is beneficial, it negatively affects the piglet's own IgG production (Klobasa *et al.,* 1981). Thus, resulting in a negative relationship between the acquisition of passive immunity through colostrum uptake and active immunity, being initiated by the piglets own immune system. Piglets that did not acquire maternal colostrum even started producing own IgG after seven days (Klobasa *et al.,* 1981, Drew *et al.,* 1988).

2.3.1. Bioactive compounds of milk and their influence on barrier function

The great importance of colostrum for a healthy individual warrants an extensive analysis of milk compounds that could potentially affect the epithelial barrier function.

Ontogenesis of mammals is dependent on the mother's milk supply to the offspring during the first weeks of life. Milk is continuously secreted by the specialized alveolar epithelium in the mammary glands. Afterwards it is stored in the extendable tissue of the mammary ducts and released to the cisterns due to the contraction of muscle cells activated by the neuropeptide oxytocin (Gürtler *et al*., 2000).

Milk provides a wide variety of factors for health and development. Besides immunoglobulins, antimicrobial peptides and growth factors, milk also contains macronutrients like carbohydrates, proteins and fatty acids (Davies *et al*., 1983). The composition of milk is influenced by the animal breed and species. Moreover, external factors such as seasonal changes, the feeding system, milking frequencies and the milking system all influence the emulsions formation (Lindmark-Mansson *et al*., 2003).

Among the different components, a number of bioactive molecules have been identified which influence epithelial transport and barrier properties. For example the medium chain fatty acids, caprate and laurate, increase paracellular permeability and can therefore be clinically employed as an absorption enhancer for intestinal drug uptake (Krug *et al*., 2013,

Dittmann *et al*., 2014). The effects of laurate and caprate on barrier properties and TJ protein expression were studied by employing the colonic epithelial cell line HT-29/B6. In these studies caprate and laurate induced a reversible reduction of barrier properties, which could be identified as a pure paracellular effect for both compounds. Further experiments revealed a selective effect on single TJ proteins, namely tricellulin and claudin-5. Whereas a decrease of claudin-5 correlated to the drop of TER (Amasheh *et al*., 2005), tricellulin is known to seal the epithelial barrier in sites of tricellular cell contact (Krug *et al*., 2009). The specific local permeability induced by caprate in tricellular cell contacts was visualized by the development of a novel molecular imaging strategy analyzing the translocation by means of apical labeling with sulfo-NHS-SS-biotin (Krug *et al*., 2013).

Subsequent to the published study of caprate effects on the intestinal epithelium, the effects of laurate have been analyzed. Laurate also naturally occurs in porcine, cow and human milk (Moltó-Puigmarti *et al*., 2011; Zentek *et al*., 2011), as well as in a variety of plant oils such as coconut and palm kemel oil (Gurr *et al*., 2002) which demonstrates biocompatibility of the compound. In Caco-2 cells, laurate induced a rapid decrease in TER and an increase in permeability for solutes such as fluorescein and [^{14}C]-mannitol (Lindmark *et al*., 1998). For further analysis, TER measurements and two-path impedance spectroscopy were carried out (Dittmann *et al.,* 2014). Laurate induced a time-dependent reduction of TER, which was reversible after wash out and could directly be linked to an exclusive effect on the paracellar resistance *via* two-path impedance spectroscopy (Krug *et al*., 2013). To evaluate the extent of the paracellular barrier regulation permeability measurements for markers of different sizes were also carried out. Furthermore, expression and localization analysis of TJ proteins were performed to align the functional results with molecular proof. In this set of experiments, an uncoupling of the effects with caprate was observed, as the increased paracellular permeability was limited to fluorescein (330 Da). In comparison, the permeability for larger molecules such as sulpho-NHS-SS-biotin (607 Da) and 4 kDa FITC-dextran was not affected. It was concluded that the effect on TJ structure was mediated by selective removal of the sealing TJ protein claudin-5 from the TJ without an effect on tricellular cell contacts (Dittmann *et al*., 2014). These findings are in accordance with previous functional characterization of claudin-5 (Amasheh *et al.,* 2005).

Furthermore, milk component whey has also been shown to influence intestinal epithelial properties through the transforming growth factor-β (TGF-β) (Hering *et al*., 2011). It has been proven to uphold barrier function even after enterohemorrhagic *Escherichia coli* infection (Howe *et al*., 2005). Currently it is even being used as a therapeutic drug against Crohn's disease (Fell, 2005), which is a chronic inflammatory disorder where the body attacks the intestinal system. TGF-β has also been shown to positively influence the immunological response after antigen contact and therefore preventing allergies (Verhasselt *et al*., 2008).

Hering showed that TJ protein claudin-4 can be regulated by TGF-β. This interaction is potentially dose-dependent. Moreover the epithelial barrier function is increased, giving this result a functional importance as well (Hering et al., 2011).

Altogether milk is an emulsion of various bioactive compounds, which, each in their own, might influence the epithelial barrier function. Therefore the first part of this thesis focused on the overall effect of milk on TER and paracellular permeability.

2.3.2. Peyer's patches as the intestinal sensors for the immune system

For the intestinal immune system, PP play a major role for recognition and uptake of antigens from the lumen. They are a major part of the gut-associated lymphoid tissue (GALT) (Jung *et al*., 2010). Even though their function was unknown until 1922, when Kumagai detected *Mycobacterium tuberculosis* inside the PP (Kumagai, 1922), they were first described in detail by Johann Peyer in 1677 and named accordingly.

The follicle-associated epithelium (FAE) is responsible for the uptake and transport of antigens to the underlying tissue. It forms a boundary separating lumen content and immune cells. Transport of antigens through FAE of PP epithelium is a key step for initiation of immune reactions.

In porcine intestine, about 20 disk-like discrete PP are located in the jejunum, averaging at a size of three to eight centimeters. Moreover, there is one large continuous PP in the ileum, which can be up to three meters long. The spiral colon has approximately ten irregular shaped patches (Kararli, 1995).

Microscopically, PP are covered by the FAE, which contains microfold cells (M cells). M cells derive from common epithelial cells and are specialized in antigen translocation and presentation to the underlying lymphocytes (Owen, 1977; Wolf *et al*., 1981; Onishi *et al*., 2007). Moreover, FAE lacks the subepithelial myofibroblast sheath, and the basal lamina of FAE is more porous compared to villus epithelium (Takeuchi & Gonda, 2004). Due to fewer goblet cells being present in the FAE (Onori *et al.,* 2001) the mucus covering the epithelium is thinner than normal (Ermund *et al*., 2013). The presentation of antigen induces activation of B and T lymphocytes in the follicular area of PP (Gebert *et al*., 1996).

The PP is organized in lymphoid follicles right underneath the FAE, each having a subepithelial dome, also called the corona (**Fig. 5**). While the follicle itself contains a germinal center with B lymphocytes, dendritic cells and macrophages, the corona is home to B lymphocytes, T lymphocytes, dendritic cells and macrophages. Antigen contact can either be directly initiated by M cells sampling from the gut lumen or indirectly by transcellularly extending dendrites (Lelouard *et al.,* 2011).

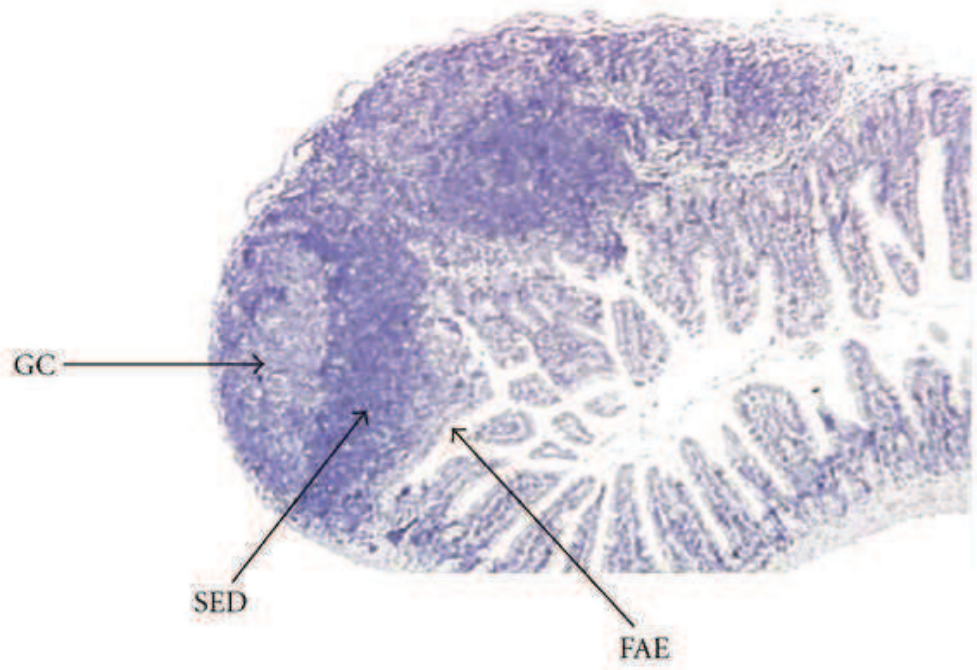

Figure 5: Histological features of Peyer's patches. Peyer's patches are located within the intestinal submucosa of the distal small intestine. Histologically, they can be structured into a germinal center (GC), containing B lymphocytes, dendritic cells and macrophages, a covering follicle-associated epithelium (FAE) containing enterocytes and M cells, and a layer in between, the subepithelial dome (SED) made up of B and T lymphocytes, macrophages and dendritic cells (Jung *et al.,* 2010).

Functional comparison of transepithelial barrier function employing rabbit PP and villus epithelium revealed a markedly higher TER in FAE compared to neighboring villus epithelium (Brayden *et al*., 1994). Claudin-2, -3 and -4 expression in murine FAE could also be proven in 2002 via immunohistochemistry (Tamagawa *et al*., 2003). However, a quantitative and qualitative comparative approach employing neighboring villus epithelium has not been investigated to date and is the focus of the second part of this thesis.

Chapter 3: Aims and objectives of this thesis

The protein family of claudins has recently been identified as the main functional component of epithelial TJ. This underlines the importance of further research directed at this specific group of membrane proteins. This thesis is focused on two separate objectives:

1. **Challenging porcine intestinal epithelium by predigested and non-predigested milk.**

Recent research has shown that claudin expression levels and therefore paracellular permeability can be influenced by incubation with different absorption enhancers. It has been concluded that medium-chain fatty acids laurate (Dittmann *et al*., 2014) and caprate (Krug *et al*., 2009) influence the TER, the paracellular permeability and the expression of tricellulin and claudin-5. These findings prompt a number of basic questions regarding the intestinal epithelial physiology including the possible effects of milk on transport and barrier properties, and TJ protein expression levels. Hence an intra-species model was chosen to further evaluate this issue.
In this study, a series of Ussing chamber experiments were performed, while paracellular flux measurements were also carried out. Small intestinal tissue of piglets at three different ages was incubated with a 50 % predigested and 50 % non-predigested milk-buffer mixture. Incubation samples were taken and molecular analysis was carried out, by immunoblots and immunohistochemistry.

2. **Functional and molecular characterization of the porcine and murine tight junction within small intestinal Peyer's patches.**

Minimal information regarding functional and molecular PP barrier properties is available. In comparison general expression and localization of TJ proteins in different locations of intestinal villus epithelium has been investigated. However, information about specific expression levels of tight junction proteins in FAE is still scarce. The functional relevance and contribution of M cells within FAE is evident and therefore needs to be addressed. Previous studies have established functional differences between both epithelia (Brayden *et al*., 1994), which could not be linked to specific protein expression patterns.
This part of the thesis aimed at a detailed molecular and functional barrier analysis of the porcine and murine PP FAE in comparison to villus epithelium.
A set of Ussing chamber experiments was carried out, by using villus epithelium and FAE. Functional and structural analysis of immunoblotting, RT-PCR and immunohistochemistry were conducted.

Chapter 4: Milk induces a strengthening of barrier function in porcine jejunal epithelium *in vitro*

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Chapter 6: Molecular characterization of barrier properties in follicle-associated epithelium of porcine Peyer's patches reveals major sealing function of claudin-4

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Chapter 7: Discussion

Porcine milk induces a strengthening of barrier function in porcine jejunal epithelium *in vitro*

The first part of this thesis focused on the effects of milk on jejunal barrier function employing an intra-species model incubating porcine jejunal epithelium with porcine milk. The question of whether milk has a barrier strengthening effect or may enhance the absorptive capacity of jejunal epithelia was to be answered.

In this study incubation of porcine jejunal epithelium with either predigested or non-predigested milk led to a significant increase of TER across three tested age groups. However, the permeability for the paracellular marker sodium fluorescein was not significantly changed (see **Fig. 1**, p. 22, **Fig. 2**, p. 23). While quantitative analysis of TJ protein expression levels for claudin-1, -2, -3 and -4 revealed no significant difference (see **Fig. 3**, p. 24), immunohistological assessment revealed a change of location for claudin-3 and -4. Claudin-3 was internalized and could be detected in intracellular vesicles, whereas claudin-4 showed reduced signals in apicolateral membranes and a translocation to subjunctional compartments of the cells (see **Fig. 4**, p. 25).

During digestion, different digestion enzymes and fluids are secreted. These physiological reactions to the intake of food lead to a dilution of the ingesta, which has to be noted when studying effects of possible nutritional factors on the intestinal epithelial barrier. While performing an initial investigation studying IPEC-J2 cell monolayers grown on permeable inserts and different milk concentrations, it became apparent that concentrations higher than 50 % milk resulted in increased standard deviations. This was attributed to insufficient buffer capacity of the experimental solution. In accordance with previous research (Ma & Verkman, 1999) further experiments were conducted with 50 % milk-buffer mixtures. Furthermore, an established predigestion protocol was used to account for possible effects due to the digestive processes on bioactive molecules that are contained in milk. Through addition of amylase, lactase and lipase the most prominent gastric and jejunal enzymatic reactions could be mimicked (Yao *et al*., 2010).

The most substantial results were obtained in the 2 month age group where TER was increased after 30 and 150 minutes of incubation. The results of the younger age group (1 month) were similar, but only showing an increased TER after 30 minutes. Interestingly, this rapid effect could also be reproduced with piglets of two weeks of age. As older age groups are commonly chosen for Ussing chamber experiments due to an advanced epithelial maturation, a use of younger piglets is appropriate when studying the presented topic. Younger piglets physiologically consume milk until weaning and would serve as an ideal model, even for the human gastrointestinal tract. However, tissue samples of younger animals could not be stripped (removal of serosal and muscular layer) without degrading epithelial integrity, therefore the molecular analysis was focused on the 2 month age group of piglets.

Bioactive compounds in porcine milk

Since milk is a very heterogenous emulsion of different energy carriers, hormones, growth factors and other bioactive compounds (Lindmark-Mansson *et al*., 2003), it is difficult to attribute specific experimental results, using whole milk, to one compound.

Previously the medium-chain fatty acids, laurate (Dittmann *et al.,* 2014) and caprate (Krug *et al.,* 2013) have been proven to reversibly impair the epithelial intestinal barrier in *in vitro* experiments using different cell culture setups. In the study of Krug *et al.* (2013) caprate incubation was carried out using 3 mM, 10 mM and even 30 mM. Caprate concentration in porcine milk used in this study could be measured at 2.89 ± 0.13 mM, which is of similar range and making it plausible that an effect on barrier function could be expected. Laurate on the other hand, has been shown to significantly decrease barrier function at 3.5 mM (Dittmann *et al*., 2014), making it a better candidate for potentially affecting the epithelial barrier function. Within this study, comparable concentration of laurate (1.15 ± 0.11 mM) could be detected in porcine milk. However, due to the gastrointestinal secretion of digestion fluids dilution of the ingested milk has to be assumed and therefore only 50 % milk-buffer mixtures were applied, resulting in lower concentrations of the fatty acids. Furthermore, incubation with both fatty acids led to a significant increase of paracellular marker molecule permeability (Krug *et al*., 2013; Dittmann *et al.,* 2014). While laurate incubation induced increased permeability only for fluorescein (330 Da), caprate enabled even bigger marker molecules, such as 10 kDa FITC-dextran to be transported via the paracellular route. Since an increased TER and a stable permeability for fluorescein were recorded in the current experimental setup, it can be assumed that either (a) caprate and laurate do not significantly influence epithelial barrier function *in vivo,* or (b), that the effects of both fatty acids are masked by other potent milk compounds, where the latter is more plausible.

Two major protein fractions are contained in milk: (1) whey: a mixture of proteins, that remains after cheese production, (2) casein, the major cheese component. In porcine milk the percentage of both protein fractions is greatly dependent on the phase of lactation. While casein increases from 9 - 32 % in colostrum to 30 - 45 % 24 h after birth, whey protein amount drops from 90 % to 70 % (Theil & Hurley, 2016). Both protein fractions are contained evenly in mature milk (Csapo *et al.,* 1996).

TGF-β is a whey protein component and has recently been shown to increase barrier function due to upregulation of tight junction protein claudin-4 in human colonic HT-29/B6 cells (Hering *et al*., 2011). As a multifunctional cytokine, TGF-β is heavily involved in the regulation of inflammatory processes (Letterio *et al*., 1998), in the protection against infection (Oddy *et al.,* 2003) and in the ignition and maintenance of oral tolerance against food derived antigens (Soyoung *et al.,* 2013). Even though the increased barrier function could be reproduced in this study, claudin-4 expression levels remained unchanged.

Lactoferrin (LF) is another multifunctional whey protein in porcine milk (Masson *et al*., 1970; Chu *et al.,* 1993). LF is structurally closely related to the ion transporting protein transferrin

and may also bind and transfer iron molecules (Metz-Boutique *et al*., 1984). While a higher concentration of LF could be detected at the beginning of lactation, it usually declines throughout the rest of the lactation period (Elliot *et al*., 1984). As the milk used in this study was taken during the first days of lactation, a higher concentration of LF can be assumed. LF has bacteriostatic and immunologic activity, making it another protein of interest, when looking at epithelial barrier function under the influence of milk. Through binding of iron, LF has the ability of withholding the element from iron-deficient bacteria (Weinberg, 1984). Moreover, it might be able to destabilize the bacterial cell wall (Ellison *et al.,* 1990) or block the microbial metabolism (Arnold *et al*., 1982). Hence, an antibiotic effect within the mother's mammary gland and the gastrointestinal tract of suckling offspring is plausible. LF may also support the bactericidal function of neutrophils because it withholds iron and therefore inhibits growth and induces death of internalized bacteria (Bullen & Armstrong, 1977). It also provides iron for the production of free radicals, which could increase phagocytotic activity and therefore improve the immunological defense (Lima & Kierszenbaum, 1987). LF is considered to be part of the innate immune system as it binds to specific receptors found on enterocytes (Gíslason *et al*., 1993; Suzuki *et al*., 2005) and is one of the first defense systems against possible harmful agents. Kirkpatrick *et al.* (1971) showed that LF inhibits growth and development of various bacteria, viruses, parasites and fungi. Recently it has been shown that dietary bovine LF indeed increases intestinal crypt cell proliferation in neonatal piglets (Reznikov *et al.,* 2014). The effect of LF on epithelial barrier integrity of Caco-2 cell layers revealed a strengthening of epithelial barrier function after incubation with early-lactation extracted LF (Anderson *et al*., 2017). This could be linked to a LF induced reduction of interleukin-8, which is known to decrease barrier function. These results could not be reproduced with mid-lactation LF, which might be due to missing angiogenin, which is known to suppress inflammatory responses (Anderson *et al*., 2017). Therefore, a possible strengthening influence of LF on the epithelial barrier function is probable, regarding current research results that point in this direction (Anderson *et al*., 2017).

Long term incubation experiments employing milk and porcine intestinal epithelium have not been carried out to date, since the viability of porcine tissue in an Ussing chamber setup is limited. Nevertheless, results from similar studies employing porcine cell lines and single milk components have been reported. Amino acids glycine and L-glutamine enhance the epithelial barrier function and reduce paracellular permeability when applied in physiological concentrations (Li *et al.,* 2016, Wang *et al*., 2016). Furthermore, application of glycine led to increased expression levels of claudin-7 and the scaffolding protein ZO-3. Molecular effects of incubation with L-glutamine led to an increased expression of scaffolding proteins and claudins. Combining these results, both amino acids improve epithelial integrity through TJ protein abundance and relocation from the cytosol to the cellular membrane. As both amino acids are contained in milk, they might have also influenced barrier integrity in the current study performed.

Considering the composition of milk, a rather unspecific covering of the epithelial monolayer might be possible. Porcine milk contains about 5 - 6 % of fat, depending on the phase of

lactation (Csapó *et al*., 1994). Due to the structure of fat molecules and the composition of the cellular membrane an interaction between both would be possible. A covering of the paracellular opening would indeed lead to an increased barrier function, as the paracellular route is blocked. On the other hand, a specific interaction of caprate and lipid rafts has been indicated by Sugibayashi *et al*. (2009) in Madin Darby Canine Kidney (MDCK) cells. In this study claudin-4, -5 and occludin showed increased Triton X-100 solubility after incubation with 10 mM caprate, leading the authors to conclude that a displacement of these TJ proteins via lipid rafts was the reason for the decreased epithelial barrier function. Lipid rafts are floating aggregations in the cellular membrane, made up of a high content of sphingomyelins, glycosphingolipids and cholesterol (Thomas *et al*., 2004). As proteins can also be localized within these lipid rafts a possible connection to regulatory functions via lipid rafts might be feasible.

Due to the multiple bioactive compounds in milk, the increased TER cannot be safely linked to one single ingredient. However, numerous studies have shown, that milk improves and accelerates gut maturation in human (Jacobi & Odle, 2012; Garcia *et al*., 2013) and piglet (Bjornvad *et al.,* 2008; Møller *et al*., 2011; Pieper *et al*., 2015; Berding *et al*., 2016) and therefore the integrity of the intestinal epithelial barrier. As a tight intestinal barrier is important to regulate the contact of antigen with parts of the intestinal immune system, early gut maturation is advantageous.

The influence of digestive processes

In this study a predigestion protocol was used to mimic gastrointestinal conditions and account for the specific physiological processes that happen during the passage of milk through the gastrointestinal tract. Digestion processes were simulated using the established protocol by Yao *et al.* (2010). As comparable results were obtained while performing incubation experiments with predigested and non-predigested milk, it can be concluded that the barrier function is presumably influenced by bioactive compounds which are not affected by the digestion process. The addition of bile would present an interestingly new aspect, since its main function is the emulsion of fatty acids, making them accessible for lipases and therefore induces increased absorption (Maldonado-Valderrama *et al*., 2011).

Effects of milk incubation on tight junction proteins

Since the effects of milk incubation on porcine intestinal epithelial barrier function were limited to an increased TER, while no permeability changes for sodium fluorescein (330 Da) were detected, further interest was oriented towards single claudins which are known to be responsible for ion transport.

Claudin-1 is typically characterized as a sealing claudin (Inai *et al*., 1999), which is usually located in the apical junction, but sometimes can also be detected throughout the lateral intercellular junctions or even the basal membranes of the cell (Coyne *et al.,* 2003). It has been shown that claudin-1 interacts with claudin-3 in the airway to form a tighter barrier and

reduce permeability (Coyne *et al*., 2003). The knock-out of claudin-1 in mice leads to lethal water loss through the skin (Furuse *et al.,* 2002).

Claudin-2 is classified as a pore-forming claudin, as the presence of claudin-2 transforms tight epithelial junctions into leaky ones, as shown by Amasheh *et al.* (2002). Therefore, it is typically expressed in epithelia that absorb and secrete profusely, for example the proximal tube of the kidney (Muto *et al*., 2010) and the small intestine (Markov *et al*., 2010). Overexpression of claudin-2 also led to the increased absorption of cations (Amasheh *et al.,* 2002) and therefore increased permeability. Moreover, claudin-2 functions as a paracellular water channel (Rosenthal *et al*., 2010).

Sealing tight junction protein claudin-3 has been classified recently. Employing a kidney model, claudin-3 decreases the permeability of paracellular markers and ions, while the water flux remained unaffected (Milatz *et al*., 2010). Furthermore, TER was elevated after introducing claudin-3 into the tight junction of MDCK II cells (Milatz *et al*., 2010). Interaction of claudin-1 and claudin-3 has been proven in 2003 (Coyne *et a*l). Claudin-3 is also expressed in organs requiring a tight epithelial barrier such as the gall bladder and the tighter segments of the nephron (Kiuchi-Saishin *et al.,* 2002; Laurila *et al*., 2007).

Claudin-4 has been shown to act as either a sealing or pore-forming claudin. In MDCK-II overexpression of claudin-4 leads to a reduction of cation permeability and a more complex structure of TJ strands. The permeability for markers such as fluorescein and mannitol remained unchanged (Van Itallie *et al.,* 2001). A knockdown of claudin-4 in cancerous bladder cells also resulted in manipulated epithelial integrity, as the TER was reduced (Boireau *et al*., 2007). An interaction between claudin-4 and claudin-8 is necessary to form a chloride channel in the kidneys collecting duct (Hou *et al.,* 2010), which also enhances the paracellular permeability. However, as previously mentioned, the whey component TGF-β leads to an increased TER and strengthens the epithelial barrier function (Hering *et al.,* 2011). Claudin-4s diverging functions might be the result of specific interactions of different claudins or milk components and claudins.

In this thesis, the effects of milk incubation on claudin expression levels were studied. Samples of both tested age groups (1 month and 2 months) revealed no change in expression for the tested claudins-1, -2, -3 and -4. Claudin-3 and -4 were selected for immunohistochemistry since both claudins have been shown to influence ion permeability without affecting permeability for macromolecules (Milatz *et al.,* 2010, Van Itallie *et al*., 2001) and provide reliable signals via immunohistochemistry to analyze possible altered locations (Schierack *et al.,* 2006). In control stainings claudin-3 and -4 were solely localized in the paracellular regions. After incubation with either predigested or non-predigested milk claudin-3 was internalized and claudin-4 was moved to subjunctional compartments. As claudin-3 and -4 are usually classified as sealing claudins (Hering *et al*., 2011, Milatz *et al.,* 2010) it appears contradictory that they are removed from the TJ. However, these results are in accordance with previous studies focusing on the rapid effects of caprate and laurate (Dittmann *et al.,* 2014, Krug *et al*., 2013), where the reversible removal of single TJ proteins could be shown. While reversibility was not tested in this thesis, it would be of great interest

to find out whether the functional changes and the relocation of TJ proteins due to milk incubation could be reversed.

A possible explanation for the observed quick effects could be the removal of claudins *via* lipid rafts (Sugibayashi *et al*., 2009) or intracellular signal cascades. Furthermore, even a direct interaction of milk compounds and parts of the cellular membrane is possible as was shown by Li *et al* (2008) for cytokines tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ). TNF-α is one milk compound (Rudloff *et al*., 1992) with multiple functions involved in inflammation. It is mostly produced by macrophages (Olszewski *et al*., 2007) and released after recognition of lipopolysaccharides or other antigen. TNF-α induces many signs of inflammation: heat, swelling, redness, pain and loss of function. In addition, it increases the expression of adhesion molecules to attract neutrophils or other immune cells to local centers of inflammation (Mackay *et al.,* 1993). IFN-γ is usually secreted by T helper cells, but is also contained in milk (Ewaschuk *et al*., 2011). IFN-γ supports and stimulates the cellular immune system through activation of macrophages (Bastos *et al*., 2007). Moreover, IFN-γ regulates the intracellular process of phagocytosis and enhances the production of reactive oxygen species (Fávaro *et al.,* 2014). Regarding the TJ, it has recently been shown that IFN-γ and TNF-α synergistically influence the epithelial barrier function through disruption of the TJ structure (Li *et al.,* 2008). Both cytokines lead to a changed lipid composition of the cellular membrane, therefore affecting TJ structure and enabling the removal of TJ protein occludin *via* lipid rafts. At the same time TER was decreased, while the permeability for FITC-dextran increased. A comparative effect of both cytokines in the current approach might have also influenced the results of the current study performed.

Overall it is important to note that an intra-species model was used in this approach and the application of milk instead of single compounds could also impact different regulatory mechanisms. Furthermore, the direct transfer of research acquired in cell culture setups is debatable when looking at *in vitro* studies, which impedes direct comparisons of the different models. Regarding everything that has been said above it can safely be assumed that other milk compounds may affect the epithelial barrier integrity. Those effects might even be divergent, but still accumulate to an overall strengthening effect.

Barrier properties in murine and porcine PP

The second part of this thesis focused on the functional and molecular characterization of the porcine and murine FAE in PP. Electrophysiological measurements displayed higher TER for PP tissue when compared to neighboring VE (see **Fig. 1A**, p. 41; **Fig. 2**, p. 32).The paracellular permeability was significantly reduced for smaller marker molecules like fluorescein (see **Fig. 1B**, p. 41) and bigger molecules such as 4 kDa and 20 kDa FITC-dextran (see **Fig. 4**, p. 33). One-path impedance spectroscopy was performed and found increased R^{epi} and R^{sub} for murine PP (see **Fig. 3**, p. 33). Densitometric analysis of immunoblots using murine samples displayed a decrease in signal for claudin-2 and -7, whereas the signal for claudin-8 increased (see **Fig. 5**, p. 33). Porcine tissue exhibited decreased expression levels for claudin-3, -5 and -8 (see **Fig. 3**, p. 42). Regarding the

specific morphology of the epithelial layer covering PP, a surface correction had to be performed for comparison of VE and PP data. While the VE is characterized by typically meandering epithelium, PP is covered by FAE and VE (for comparison see **Fig. 1**, p. 32). FAE forms dome-like structures covering lymphoid follicles, therefore less intercellular connections are contained per square centimeter, affecting the direct comparison of TJ protein content between both tissues. After correcting the data with the calculated surface correction factor, murine claudin-1, -4, -5 and -8 and porcine claudin-4 were significantly elevated. Immunohistochemical detection of claudins revealed apicolateral localization of claudins in PP as well as VE in both species, confirming the functional localization of all tested claudins in FAE (see **Fig. 4**, p. 43, **Fig. 6**, p. 34).

The intestinal epithelium has the difficult task of distinguishing between common food ingredients and potentially harmful pathogens, as the ingesta are being transported through the gastrointestinal tract. While undifferentiated uptake of pathogens must be inhibited, absorption of nutrients needs to be ensured at the same time.
Epithelial transport routes can be divided into the transcellular and the paracellular pathway. While specific transporters are localized in the apical and basolateral membranes of epithelial cells enabling transcellular transport, the paracellular transport is less specific, and controlled and regulated by the TJ (Bruewer & Nusrat, 2006). The focus of this study was the characterization of the paracellular transport route in porcine and murine PP.
PP are part of the gut-associated-lymphoid tissue (Jung *et al*., 2010), representing the immune system within the *lamina propria* of the gut. The FAE covers the underlying lymphoid follicle and directly controls the contact of gut content and immune system. FAE is made up of enterocytes, but also contains specialized cells, which are known as M cells. M cells can directly sample antigen from the gut lumen and present it to the underlying PP (Owen, 1977; Wolf *et al*., 1981; Onishi *et al*., 2007). Furthermore, dendritic cells are able to reach out through the epithelial monolayer and extend their dendrites all the way into the gut lumen to bind antigens (Lelouard *et al.,* 2011). This alternative route is specific for FAE, ensuring a quick and easy interaction between antigen and immune system.
The functional results obtained in this study underline the importance of the TJ structure in FAE. As accidental sampling of antigen needs to be minimized, a tight paracellular barrier in the epithelium covering PP is an important requirement for the controlled maturation of the immune system and the initiation of immune reactions. Conveniently, the paracellular permeability which represents paracellular transport is limited for molecules of various sizes. The paracellular markers fluorescein and FITC-dextran (4 kDa, 20 kDa) provided important information on the size-dependent preference of the FAE. In conjunction with these results it can safely be concluded that the permeability for larger and smaller molecules is greatly reduced. Furthermore, even the R^{sub} showed significant increase in PP tissues. As FAE lacks the subepithelial myofibroblast sheath and has a more porous basal lamina compared to villus epithelium (Takeuchi & Gonda, 2004), this might be attributed to a physiologically

increased thickness of the subepithelial tissue layer as shown in tissues with chronic subepithelial immune cell infiltrations (Juric *et al.,* 2013).

Even though every needed cell type is located within the *lamina propria* at birth, the PP only fully matures at the age of three weeks, as reported for pigs by Makala *et al*. (2000). A controlled and regulated interaction of antigen and gut-associated immune system can take place from this point on. This supports the relevance of the FAE even more, because the paracellular barrier tightly controls interaction between the underlying PP and antigen. However, it has been shown that interactions between pathogenic organisms and the host can lead to an alternative uptake route, mediated by dendritic cells. These cells can actively open TJ, extend dendrite into the gut lumen and bind available antigen, avoiding the physiological route of uptake via M cells. The intestinal barrier integrity is not impaired due to dendritic cells expressing TJ proteins that close the formed gap within the otherwise secure barrier (Rescigno *et al*., 2001). As the development of PP is critically influenced by the interaction of pathogen and the immune system (Barmann *et al*., 1997), a regulated contact must be possible.

The current results were acquired using tissue from seven week old piglets. PP and other parts of the porcine immune system are morphologically fully developed at this age. As piglets are usually weaned at three to four weeks after birth, the challenging post-weaning stage was already completed for the sampled animals and results can be assumed to be transferable to adult individuals as well.

Results from previous studies employing rabbit tissue (Brayden *et al*., 1994) are in accordance with the outcome of the current study. The detection of a series of TJ proteins proved the expression of claudins in porcine and murine FAE, as well as the paracellular localization in the apicolateral part of the cellular membranes. As claudins determine the paracellular permeability for molecules and ions (France & Turner, 2017), an increased expression of at least some members of the claudin family was expected.

To allow proper comparison of immunoblotting results, the difference in morphology of the epithelial cell layer covering PP and the VE had to be studied. Through geometrical approximation, a correction factor was calculated, representing the higher epithelial cell number and therefore portion of TJ proteins in whole protein samples obtained from VE. For simplicity in calculations a round shape PP was estimated. After corrections of primary immunoblot data, different claudins were shown to be highly expressed in PP tissue, when compared to VE. Murine PP revealed a stronger expression of sealing claudins -1, -4, -5 and -8, while only a higher expression of claudin-4 could be shown in porcine samples.

Claudin-1 is generally classified as a sealing claudin (see above), which underlines its importance for epithelial barrier function in the gut. Interestingly, common peanut allergens Ara h 1 and Ara h 2 have been shown to disrupt the epithelial TJ in Caco-2 cells (Price *et al*., 2014). Bypassing the common route of pathogens *via* M cell transcytosis and therefore enabling allergic reactions, showing that further localizations of TJ might be important when looking at immunological issues. Moreover, dendritic cells can express claudin-1, maintaining

a stable barrier, while sampling antigens from the gut (Rescigno *et al*., 2001) supporting the relevance of claudin-1 for the intestinal epithelial barrier integrity.

Claudin-5 is usually also expressed in endothelial cells, making it an important candidate for blood-brain barrier research (Amasheh *et al.,* 2005). It has been shown to selectively decrease the paracellular conductance of TJ in MDCK II cells (Wen *et al*., 2004), therefore also classifying it as a sealing claudin.

Functional data on the relevance of claudin-8 was also studied in MDCK II cells, revealing an increased paracellular barrier inhibiting the passage of cations. However, permeability for anions or neutrally charged molecules was not changed (Yu *et al*., 2003).

As already discussed above an upregulation of claudin-4 solely increases the TER and reduces cation permeability (Van Itallie *et al*., 2001). In conjunction with claudin-8 chloride channels can be formed (Hou *et al*., 2010). However, this also increases the permeability. Therefore, claudin-4 can be classified as a claudin with ambiguous function.

The overexpression of claudin-1, -4, -5 and -8 in murine and porcine tissue samples of PP are in accordance with the functional results obtained in this study. When compared with VE there is an increase in TER and also limited paracellular permeability. Differences across species as compiled here for pigs and rats allows for further research being done on other laboratory animals such as mice and suggests that even more claudins could be responsible for the presented outcome. In 2001, Onori *et al.* focused on the morphology of rats PP, concluding that M cells within the FAE could not be identified *via* morphological criteria, such as microvilli length and lysosome number. Nevertheless, a colocalization of epithelial cells with short and disorganized microvilli and underlying lymphocytes could be confirmed, raising the question of whether M cell morphology dynamically reacts to stimuli or the cell cycle. As previously used M cell marker cytokeratin 18 (Gebert *et al*., 1994) did not show specific signals in our samples with rat and porcine tissue (data not shown), the need of a reliable M cell marker system still remains. Specific claudin combinations in between M cells and VE are a likely candidate since functional combinations of claudins are already well known in other tissues such as the airway (Coyne *et al*., 2003) and kidney (Hou *et al*., 2010). One of these proteins could be claudin-4 as it was verified to be elevated in FAE in both pigs and rats. A previous study that was focused on mouse PP also revealed an increased expression of claudin-4 within the FAE (Tamagawa *et al*., 2003). Furthermore, claudin-4 was mostly associated with the apical dome region of the FAE. Combined with the current data, the hypothesis that claudin-4 might help maintain the structural morphology of the dome region and could also influence the uptake of antigens (Tamagawa *et al.,* 2003) is strongly supported.

The importance of rat and pigs used as laboratory animals in translational biomedicine

While the obtained results exclusively characterize TJ protein expression and regulation in rats and pigs, the question of whether these findings allow insight into human gastrointestinal physiology remains. Ever since the ancient Greeks animal models were used to study physiological and pathophysiological processes, as well as practice therapeutic treatments.

Historically used as one of the first laboratory animals, the rat was first utilized for behavioral analysis in 1903 (Watson). Later, usage broadened into other fields of researches and in 2004 most parts of the rats' genome were sequenced (Mullins & Mullins, 2004). Due to their bigger body size physiological measurements on rats are preferable and easier to carry out, when compared to mice. Furthermore, certain experimental techniques enabling *in vitro* assays, as for example the Ussing chamber technique employed in this study, are not limited due to the size of the animal. Moreover, the rat is evolutionary closer to the human than the mouse, as their development is millions of years apart and they do show obvious physiological differences (Iannaccone & Jacob, 2009). Rats are currently used for research in cardiovascular, behavioural and neurological sciences. Moreover, research focusing on pathological growths such as cancer is commonly carried out in rats (Iannaccone & Jacob, 2009). The pig offers more insight due to the even closer evolutionary relationship between pigs and mankind. Furthermore, it is of human size, has similar physiology, a larger number of offspring and relatively similar disease progression (Lunney, 2007). Even direct organ transplantation between both species might be possible in the future (Ibrahim *et al.,* 2006). The pig is preferably used for comparison studies on the gastrointestinal tract and immunological problems (Pond & Houpt, 1978; Rothkötter *et al*., 2002). Currently, piglets are commonly used to model gastrointestinal processes in infants (Moughan *et al.,* 1992; Alizadeh *et al*., 2016). Postnatal developmental changes and development of the gastrointestinal tract are more closely resembled by the piglet, when compared to laboratory rodents (Buddington & Sangild, 2012; Heinritz *et al.,* 2013). This highlights the importance of the obtained results, since it could be shown that the natural source of nutrition enhances the gastrointestinal barrier and therefore provides protection against the entry of potentially pathogenous organisms. This is in accordance with previous results, showing that milk accelerates gut maturation in humans and piglets (Jacobi & Odle, 2012; Bjornvad *et al.,* 2008; Pieper *et al*., 2015). Another experimental setup employing formula would be of great interest, especially regarding the effects of separate components on the epithelial barrier. Since formula feeding in humans is associated with increased infectious morbidity, childhood obesity, diabetes mellitus, leukemia and sudden infant death syndrome (Stuebe, 2009), a closer look might be necessary to further evaluate the aftermath of formula feeding in piglets when compared to milk.

As the pig is a commonly used laboratory animal for immunological studies (Rothkötter *et al*., 2002) it was rather surprising that almost no functional and molecular data on the epithelial cell layer covering PP was found. Due to the fact that immune reactions against obvious pathogens and common allergens are both initiated *via* PP (Jung *et al*., 2010), the regulation

of the FAE barrier integrity might give great insight into the pathophysiology of infections and allergies. As both, the murine and porcine models were employed in the studies presented in this thesis the results were quite comparable, giving insight into the molecular anatomy of the FAE. Since functional data are almost impossible to obtain in humans, these results could pave the way for further research directed at more TJ proteins as well as opening up a new field of therapeutic approaches.

Conclusion

The results presented in this thesis clarified two different aspects of intestinal barrier function and TJ protein expression. They highlight the role of single members of the claudin family for maintaining a secure barrier between the environment and the mammalian organism.

Incubation of jejunal epithelia with milk led to an increase of barrier function, paralleled by a relocalization of claudin-3 and -4 to internal vesicles and subjunctional compartments. The barrier strengthening effect physiologically may support the infant's ability to withstand the likelihood of infection underlining the importance of physiological nutrition via colostrum or milk in this crucial period of development.

Different expression levels of TJ proteins could be observed in FAE of PP when compared to VE. Electrophysiological measurements revealed an increased TER which, combined with lower permeability for paracellular marker substances, indicates overall stronger barrier properties. This was in accordance with higher expression of sealing claudins. These results could be demonstrated in rats and pigs, elucidating the molecular composition of the FAE TJ for the first time. Claudin-4 emerged to be one of the key player proteins involved in both species when it comes to upholding barrier integrity in the epithelial cell layer covering PP.

As these results were compiled employing species that usually serve well as animal models in biomedical science, the translational relevance must be highlighted, promising to allow detailed insights into similar functional and regulatory mechanisms in human physiology.

Chapter 8: Summary

Summary of the PhD Thesis:

Molecular and functional analysis of the porcine and murine small intestinal tight junction

The tight junction (TJ) surrounds epithelial cells in the upper third of the lateral cellular membrane. It connects neighboring cells and therefore supports the barrier forming of cellular monolayers (Farquhar & Palade, 1963). The TJ is formed by different proteins that are anchored within the membrane of epithelial cells. The most functional relevance is attributed to the family of claudins (Günzel & Yu, 2013). By means of different experimental methods, functional data could be obtained. This enabled a definite characterization of claudins, sorting them in different categories. Amasheh et al. proclaimed a classification into sealing and pore-forming claudins, while the rest were attributed as ambiguous functions (Amasheh *et al*., 2002, Amasheh *et al.,* 2011).
The upper epithelial monolayer in the gastrointestinal tract forms a tight barrier between the ingesta and the mammalian organism. While a sufficient nutrient supply must be guaranteed, a tight barrier against potential pathogens must be maintained. Therefore the TJ regulates the paracellular permeability (Bruewer & Nusrat, 2006), giving it a direct control over which substances can selectively enter the body. TJ proteins show different preferences for solutes, regarding charge and molecular size, classifying them as permselective (Powell, 1981).
As the permeability changes throughout the gastrointestinal tract, the claudin expression levels also vary, showing an abundance of sealing proteins within the distal part, and pore-forming claudins within the small intestine (Markov *et al*., 2010). This is in accordance with the physiological processes that occur in the different intestinal segments, as nutrient absorption takes place in the proximal part, and regulated transcellular absorption in the large intestine.
The intestinal TJ was analyzed regarding two different aspects of epithelial barrier function.

1. **Challenging porcine intestinal epithelium by predigested and non-predigested milk.**

Sufficient energy supply *via* intestinal absorption is essential within the first weeks of life. While an adequate absorption needs to be maintained, a tight intestinal barrier is required for protection against potentially pathogenous organisms. This raises the questions of if and how the intestinal epithelial barrier is regulated during milk consumption.
Employing the Ussing chamber technique, jejunal epithelium of 2 month old piglets was incubated with a 50 % milk-buffer mixture. To simulate possible effects due to digestion processes, predigested and non-predigested milk was used.

Even though the transepithelial resistance (TER) was significantly increased after 30 and 150 minutes of incubation, no changes of the paracellular permeability for sodium fluorescein could be detected. Those results could be reproduced using even younger animals (1 month or 2 weeks). Analysis of claudin expression levels revealed no changes for claudin-1, -2, -3 and -4. Relocation to intracellular vesicles and subjunctional compartments could be shown for claudin-3 and -4 *via* immunohistology.
With research to date, it is known that single milk components influence the epithelial integrity. Transforming growth factor-β (Hering *et al*., 2011) and lactoferrin (Anderson *et al*., 2017) strengthen the epithelial barrier, while medium-chain fatty acids laurate (Dittmann *et al.,* 2014) and caprate (Krug *et al*., 2013) disturb the TJ and therefore barrier properties.
Data obtained in this thesis cannot fully explain which milk component leads to the recorded results. However, it can be conducted that as a generalization, milk strengthens the intestinal barrier.

2. Functional and molecular characterization of the porcine and murine tight junction within small intestinal Peyer's patches.

PP are responsible for initiating immune reactions when confronted with potential pathogens. These are specialized areas within the mucosa of the distal small intestine and covered by the follicle-associated epithelium (FAE), which contains specialized epithelial cells (M cells). The main function of M cells is the binding and presentation of antigen to the underlying lymphoid follicle. An important requirement for this controlled interaction between antigens and the immune system is the maintenance of the epithelial barrier integrity of the FAE. Therefore the second part of this thesis focused on the characterization of the FAE.
Porcine and murine PP showed higher TER when compared to villous epithelium. This is in accordance with the lower permeability for paracellular markers fluorescein and FITC Dextran (4, 20 kDa). Expression levels of different TJ proteins revealed increased levels of claudin-1, -4, -5 and -8 in rats and only claudin-4 in pigs. The paracellular localization of claudins was confirmed *via* immunohistochemistry, and both studies highlight a major significance for claudin-4 in the FAE of PP in interspecies comparisons.
The obtained data supports the assumption that PP are clearly specialized for antigen sampling and representation of antigens to the immune system, while the TJ of the FAE provides a stronger seal of the paracellular pathway.

Zusammenfassung der Dissertation:

Molekulare und funktionelle Untersuchungen an porzinen und murinen Tight Junctions des Dünndarms

Die Tight Junction (TJ) umschließt Epithelzellen im oberen Drittel der lateralen Zellmembran. Hier dient sie der Verknüpfung benachbarter Zellen und unterstützt so die Barrierebildung der epithelialen Zellreihe (Farquhar & Palade, 1963). Gebildet wird die TJ von verschiedenen in der Zellmembran verankerten Proteinen, wobei den Claudinen die größte funktionelle Bedeutung beigemessen wird (Günzel & Yu, 2013). Anhand funktioneller Untersuchungen konnten verschiedener Claudine verschiedene Funktionen zugewiesen werden. Dies ermöglichte eine generelle Einteilung in abdichtende und porenformende Claudine, während einige Claudine aber auch beide Funktionen erfüllen können (Amasheh *et al*., 2002, Amasheh *et al*., 2011).
Im Gastrointestinaltrakt bildet die epitheliale Zellreihe die erste Barriere zwischen der Ingesta und dem Organismus des Säugetiers. Während eine ausreichende Absorption der Nährstoffe gewährleistet sein muss, muss eine Barriere gegenüber potentiellen Krankheitserregern aufrechterhalten werden. Die TJ bestimmt hier die parazelluläre Permeabilität (Bruewer & Nusrat, 2006) und steuert welche Stoffe zwischen den Epithelzellen aufgenommen werden können. Je nach den exprimierten Proteinen zeigt die TJ unterschiedliche Permeabilität für Elektrolyte, Ladungen und Molekülgrößen. Daher wird sie als permselektiv beschrieben (Powell, 1981).
Die Expression von Claudinen konnte 2010 mit den funktionellen Unterschieden der verschiedenen Darmabschnitte in Einklang gebracht werden (Markov *et al*., 2010). Während abdichtende Claudine vor allem im Oberflächenepithel des distalen Darms exprimiert werden, sind porenformende Claudine auch in den proximalen Abschnitten vorhanden. Dies unterstützt die lokal unterschiedlichen physiologischen Funktionen der einzelnen Darmabschnitte, da proximal Nährstoffe resorbiert werden und distal eine Regulation des transzellulären Transports stattfindet.
In dieser Dissertation wurde die intestinale TJ hinsichtlich zwei verschiedener Fragestellungen untersucht.

1. Die Effekte von Milch auf die intestinale epitheliale Barrierefunktion

Während die Nährstoffabsorption bereits innerhalb der ersten Lebenswochen für das Überleben neugeborener Säugetiere essentiell ist, muss gleichzeitig eine ausreichende Barriere aufrechterhalten werden um die Aufnahme von potentiell pathogenen Erregern zu minimieren. Dies wirft die Frage auf, welchen Effekt Milch auf die intestinale Barriere hat.
Mittels Ussing Kammer-Technik wurde jejunales Gewebe von 2 Monate alten Ferkeln mit einer 50 % Milch-Pufferlösung inkubiert. Um einen möglichen Einfluss des Verdauungsprozesses zu simulieren wurden die Experimente mit vorverdauter und nicht

behandelter Milch durchgeführt. Obwohl der transepitheliale Widerstand innerhalb der ersten 30 Minuten signifikant anstieg und teilweise für 2,5 h konstant erhöht blieb, konnte keine Änderung der Permeabilität für den parazellulären Marker Fluoreszein nachgewiesen werden. Diese Ergebnisse waren reproduzierbar in jüngeren Altersgruppen (1 Monat, 2 Wochen). Die Analyse der Expression von Claudin-1, -2, -3 und -4 mittels Immunoblot zeigte keine Veränderung. Mittels immunhistologischer Untersuchungen konnte jedoch gezeigt werden, dass Claudin-3 und Claudin-4 unter der Milchinkubation von der parazellulären Lokalisation unter Kontrollbedingungen abweicht. Während eine Verlagerung von Claudin-3 in intrazelluläre Vesikel gezeigt werden konnte, konnte Claudin-4 vor allem in subjunktionalen Bereichen der Zelle nachgewiesen werden.
Bisher konnte für einzelne Inhaltsstoffe der Milch nachgewiesen werden, dass sie die epitheliale Integrität beeinflussen. Während TGF-β (Hering *et al*., 2011) und Lactoferrin (Anderson *et al*., 2017) die Barriere unterstützen, vermindern die mittelkettigen Fettsäuren Laurat und Caprat die Beständigkeit der epithelialen Barriere (Dittmann *et al.,* 2014, Krug *et al*., 2013). Die in dieser Arbeit gewonnenen Ergebnisse können nicht direkt auf einen Bestandteil der Milch zurückgeführt werden, liefern jedoch eine allgemeine Aussage über den Einfluss von Milch auf die Darmbarriere.

2. Charakterisierung der epithelialen Barriere in Peyer's Patches (PP)

PP sind spezialisierte Bereiche der Schleimhaut des distalen Dünndarms, welche für die lokale Initiation der Immunantwort auf potentielle Pathogene verantwortlich sind. Sie sind mit follikel-assoziiertem Epithel (FAE) bedeckt, welches spezialisierte Epithelzellen (M-Zellen) enthält, deren Hauptaufgabe die Präsentation von Antigenen zum darunter liegenden Follikel des PP ist. Eine wichtige Voraussetzung für die korrekte Funktion von M-Zellen ist die epitheliale Integrität des FAE, deren Charakterisierung der zweite Schwerpunkt dieser Arbeit war.
Sowohl porzine und murine PP zeigten einen erhöhten TER, im Vergleich zum umgebenden resorptivem Dünndarmepithel. Dies steht in Einklang mit der geringeren Permeabilität gegenüber Fluoreszein und FITC Dextran (4, 20 kDa). Expressionslevel der verschiedenen TJ Proteine zeigten eine erhöhte Expression von Claudin-4 im porzinen Epithel, während Claudin-1,-4, -5 und -8 im murinen Epithel vermehrt nachgewiesen werden konnten. Die parazelluläre Lokalisation aller Claudine konnte mittels Immunohistochemie bestätigt werden und beide Studien zeigen, dass Claudin-4 eine speziesübergreifgende Schlüsselrolle im Bereich der Barrierefunktion des PP spielt.
Diese gewonnenen Ergebnisse stützen außerdem die bisherige Annahme, dass im Bereich der PP Antigene gebunden und dem Immunsystem präsentiert werden, wohingegen der parazelluläre Weg eine stärkere Abdichtung aufweist.

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List of publications

Publications (peer reviewed)

2017

Radloff J, Falchuk EL, Markov AG, Amasheh S
Molecular Characterization of Barrier Properties in Follicle-Associated Epithelium of Porcine Peyer's Patches reveals Major Sealing Function of Claudin-4
Front Physiol 8:579

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Selbstständigkeitserklärung

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

Berlin, den 20.02.2018 Judith Radloff

Author Contribution

(i) Radloff J, Zakrzewski SS, Pieper R, Markov AG, Amasheh S (2017). Porcine milk induces a strengthening of barrier function in porcine jejunal epithelium in vitro. Ann N Y Acad Sci. 1397:110-118.

As declared in this publication, I have performed analyses and experiments, and I wrote the article. Electrophysiology, immunoblotting, and immunofluorescence data was acquired by me personally, including data analysis.

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Immunoblotting and immunofluorescence data was acquired by me personally, including design and plan of experiments, data analysis and discussion.

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As declared in this publication, I have designed, planned, performed, and supervised the experiments and performed data analysis. Electrophysiology, immunoblotting, and immunofluorescence data was acquired by me personally, including data analysis.

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