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Developmental profile of tight junction mRNA expression in the intestine of laying hens

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Abbreviations

TJ	Tight junction
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
TAMP	Tight junction associated MARVEL proteins
kDa	Kilodalton
CLDN	Claudin
OCLN	Occludin
MD2	MARVEL D2
ZO1	Zonula occludens 1
tTJ	Tricellular tight junction
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
RPL13	60S ribosomal protein L13
TBP	TATA-box binding protein
DPH	Days post hatching
RIN	RNA integrity number
Cq	Quantification cycle
SEM	Standard error of mean
ANOVA	Analysis of variance

1. Introduction

The gut plays a central role in the digestion and absorption of nutrients and constitutes a first organ exposed to external factors influencing bird's health. The intestinal epithelial barrier acts as the first line of defense between the host and the luminal environment. Intercellular junctional complexes tightly bind the epithelial cells together and regulate the ion and molecule transport via the paracellular pathway. Tight junctions (TJs) have the main role in the intestinal barrier formation and their disruption results in a leaky gut (TOMITA et al., 2004). Therefore, TJ proteins play a critical role in maintaining physiological homeostasis and structural integrity in epithelial and endothelial tissues of vertebrate (ZIHNI et al., 2016; GÜNZEL et al., 2013; BHAT et al., 2019). The term "tight junction" stems from the incorrect assumption that the junction is static and impenetrable, as it appears on electron-microscopic images (VAN ITALLIE et al., 2019). Nowadays, literature suggests that proteins with membrane lipids contribute to the formation of TJ between two or three adjacent cells to provide a semipermeable barrier (FURUSE et al., 2002; VAN ITALLIE et al., 2004; IKENOUCHI et al., 2005; ZIHNI et al., 2016; COLLINS et al., 2013; LEE et al., 2018; VAN ITALLIE et al., 2018; TSUKITA et al., 2019; PIONTEK et al., 2020; HIGASHI et al., 2020; SAITO et al., 2021).

So far, no information is available on the developmental changes in the expression of tight junction proteins in different intestinal segments of layers, but there is only limited information on broiler chickens (COLLINS et al., 2013; OSSELAERE et al., 2013; von BUCHHOLZ et al., 2021). Broiler and laying chicken lines have different metabolisms with different metabolic requirements (BUZALA et al., 2015). Therefore, in the present research, the segment-specific and age-related changes in nine TJ gene expression in layer chickens from 1–49 days of age were examined using RT-qPCR.

2. Literature survey

2.1. Claudins and tight junction associated MARVEL Proteins:

Claudins (CLDN), derived from the Latin verb "*claudere*" for "to close", are a heterogenous group of tight junction proteins that range from 12 to 32 kDa in mass. They all share four transmembrane domains, two extracellular loops and a cytoplasmic C-terminal domain and are highly conserved proteins in vertebrate evolution (GÜNZEL et al., 2013; COLLINS et al., 2013; VAN ITALLIE et al., 2018; TSUKITA et al., 2019). By overexpression, knockdown, or knockout studies in cell lines with assessment of the change of transepithelial resistance, CLDN could be classified in barrier- and pore-forming proteins. Occludin (OCLN), MARVELD2 (MD2) and ZO1, that can be termed "tight junction associated MARVEL Proteins" (TAMP), also play a scaffolding role guaranteeing the structural integrity of the cell (GÜNZEL et al., 2013, COLLINS et al., 2013; PIONTEK et al., 2020). The expression profile of TJ proteins has been studied in the intestine of chicken embryo and broilers (COLLINS et al., 2013; OSSELAERE et al., 2013; von BUCHHOLZ et al., 2021). However, expression profile of TJ proteins in the intestinal epithelia of commercial layers has not yet been well characterized. Therefore, in the presented study, nine relevant TJ proteins were examined using the same primers and procedure as reported in a previous in-house setup (von BUCHHOLZ et al., 2021).

Permeability and ion-selectivity may not depend on the intrinsic properties of individual TJ proteins, but rather on complex heteromeric interactions between different proteins (GÜNZEL et al., 2013; PIONTEK et al., 2020). More details and characteristics of the nine TJ proteins examined in this study are addressed in the following sections.

2.1.1. Claudin 1

CLDN1 is described as a barrier-forming CLDN, expressed ubiquitously in most tissues of the body (GÜNZEL et al., 2013; PIONTEK et al., 2020). Knockout of CLDN1 in mice leads to loss of the tight junctional barrier to water and macromolecules at the stratum granulosum of the epidermis (FURUSE et al., 2002; GÜNZEL et al., 2013).

2.1.2. Claudin 3

CLDN3 is described as a barrier-forming CLDN and is expressed in various epithelia, such as respiratory, urinary, and gastrointestinal tract and in glandular tissue (GÜNZEL et al., 2013; PIONTEK et al., 2020; von BUCHHOLZ et al., 2021).

2.1.3. Claudin 5

CLDN5 is also considered a barrier-forming TJ protein, which plays an important role in sealing the endothelial TJ (GÜNZEL et al., 2013; PIONTEK et al., 2020). Its downregulation can drastically reduce the barrier integrity of epithelia (CHELAKKOT et al., 2018). It is hypothesized that there are two different versions of CLDN5, a longer and a shorter one, generated by the two start-codons within the CLDN5 gene (GÜNZEL et al., 2013).

2.1.4. Claudin 7

CLDN7 also acts as a barrier-forming CLDN, but can also form chloride-selective channels in cultured epithelial cells (GÜNZEL et al., 2013; TSUKITA et al., 2019; PIONTEK et al., 2020). CLDN7 is highly expressed at the basolateral membrane in various tissues and may play a role in regulating cell-to-cell adhesion, cell motility, and tumour progression (GÜNZEL et al., 2013).

2.1.5. Claudin 10

CLDN10 is a pore- or paracellular-channel-forming CLDN (GÜNZEL et al., 2013; TSUKITA et al., 2019; PIONTEK et al., 2020). CLDN10a-form is known to be anion-selective, while CLDN10b-form is known to be cation-selective (ZIHNI et al., 2016; TSUKITA et al., 2019). In contrast to other known pore-forming CLDNs, CLDN10 is not only charge-selective but also size-selective (GÜNZEL et al., 2013).

2.1.6. Claudin 19

CLDN19 acts as a barrier-forming CLDN (GÜNZEL et al., 2013; PIONTEK et al., 2020). It is also considered to be an example of the heterogeneous interaction between different CLDNs, as it can form a cation-pore in association with CLDN16 (GÜNZEL et al., 2013).

2.1.7. Occludin

OCLN, derived from the Latin verb "*occludere*" for "to lock", was identified in 1993, although its existence had been postulated around 30 years earlier (FARQUHAR et al., 1963; FURUSE et al., 1993). It is also called MARVELD1 (ZIHNI et al., 2016). Upon its discovery, OCLN was thought to be crucial for the formation of TJ. Later, it was found that OCLN alone does not form typical TJ strands in epithelial cell layers, and it was shown that OLCN-knockout mice could survive (TSUKITA et al., 2019).

OCLN serves as a barrier-forming and scaffolding protein (CHELAKKOT et al., 2018). OCLN-knockout and OCLN-deficient mice show a loss of gastric epithelium barrier function, suggesting that overexpression of OCLN contributes to unlimited proliferation

of cells in human lung epithelia and is a likely tumour-promotor in certain types of cancer (CHELLAKOT et al., 2018; WANG et al., 2018). Furthermore, it was reported that overexpression of OCLN improves barrier function in cell culture (SAITO et al., 2021). SHIN et al. (2018) also showed that by feeding a betaine-rich diet to laying hens increased the OCLN-expression in jejunum, indicating an increase in TJ integrity.

Although OCLN does not share genetic sequence homology with the CLDN family, it shares the same tertiary structure with four transmembrane domains and two extracellular loops, but is significantly larger (IKENOUCHI et al., 2005; LEE et al., 2018).

2.1.8. Zonula occludens 1

ZO1 was the first identified TJ protein (STEVENSON et al., 1986). In contrast to the CLDN family of TJ proteins, ZO1 is postulated to have primarily architectural significance because it acts as a cytoskeletal linker protein or scaffolding protein (GÜNZEL et al., 2013; VAN ITALLIE et al., 2004; VAN ITALLIE et al., 2018; CHELAKKOT et al., 2018).

2.1.9. Tricellulin/MARVELD2

MARVELD2 (“MAL and related proteins for vesicle trafficking and membrane link”) is also known as tricellulin (ZIHNI et al., 2016; PIONTEK et al., 2020). It was the first tricellular tight junction (tTJ) protein, identified in vertebrates in 2005, and shown to be essential for sealing tTJ (IKENOUCHI et al., 2005; VAN ITALLIE et al., 2018; PIONTEK et al., 2020; HIGASHI et al., 2020). In OCLN-knockout studies, it has been described as compensating for the missing OCLN as scaffolding TJ protein and vice versa (SAITO et al., 2021; PIONTEK et al., 2020), so there seems to be functional overlap. Tricellular tight junctions regulate cell division orientation, so they may play an important role in stem cell proliferation, e.g., in the regulation of intestinal crypt cells (HIGASHI et al., 2020).

2.2. RT-qPCR

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is a commonly used tool for quantifying differences in gene expression levels, as it combines high specificity, sensitivity, and reproducibility with relatively low expenses in labour, time and resources (ELNIFRO et al., 2000; WONG et al., 2005; TAYLOR et al., 2010; von BUCHHOLZ et al., 2021). The wide application of this methodology in diagnostics during the COVID-19 pandemic has, to some extent, made the underlying principles known to a broad public (SMITH et al., 2021).

After translation from RNA to DNA, which is performed in the same tube as the immediately following amplification in one-step-PCR (WONG et al., 2005), there are two ways to display the amplification process in real time: A non-specific one via a dsDNA-binding dye and a specific one via the use of fluorophore-linked oligonucleotides (probes) (von BUCHHOLZ et al., 2021; NAVARRO et al., 2015). The TaqMan (a portmanteau of Thermus aquaticus and the videogame character Pacman) probes are such fluorophore-linked oligonucleotides, which, in solution, do not emit measurable fluorescence because the fluorescent moiety on the 3´-end suppresses its counterpart on the 5´-end of the probe.

This equilibrium is disrupted when the DNA polymerase degrades the former during the extension phase of PCR, allowing the probe to emit fluorescence that is directly proportional to the number of synthesized PCR products. By measuring the emitted fluorescence, a direct conclusion can be drawn on the amount of PCR products (NAVARRO et al., 2015).

To further reduce workload and time required, and to consequently minimalize human error and maximalize efficiency, it is convenient to combine primers in a multiplex PCR whenever possible. However, some primers may tamper with others, form primer dimers and interfere with target amplification, and thus must be used in singleplex PCR.

Since the number of cells, quantity or quality of RNA obtained from different tissue samples and individuals can never be exactly constant, it is crucial to normalize the measured gene expression data against the expression of a set of reference genes (TAYLOR et al., 2010; SMITH et al., 2021; MITRA et al., 2016). These reference genes should show a constant expression in different tissues and age of the investigated organism (SMITH et al., 2021).

In order to ensure consistent, reproducible high-quality data from qPCR experiments, the “minimum information for publication of quantitative real-time PCR experiments” (MIQE) guidelines regarding experimental design, RNA extraction, quality and quantity

control, reverse transcription, primer design, and choice of reference genes were used in the presented study (TAYLOR et al., 2010). Applying such guidelines, MITRA et al. (2016) reported that housekeeping genes “60S ribosomal protein L13 (RPL 13)” and “TATA-box binding protein (TBP)” are superior to standardize qPCRs from chicken tissues.

3. Material and Methods

3.1. Sampling

Samples of the current experiment were obtained from an animal trial conducted at the Clinic for Poultry and Fish Medicine (license number: GZ 68.205/0121-V/3b/2018). The specific-pathogen-free White Leghorn chickens (VALO®; Lohmann, Cuxhaven, Germany) were kept in isolators and were given ad libitum access to water and a commercial diet. The body weight of the animals is shown in figure 1.

Five birds were sacrificed at eight time points (day 1, 7, 14, 21, 28, 35, 42 and day 49) post hatching (DPH). Immediately after killing, samples of approximately 2g were taken from the jejunum (slightly proximal to meckel´s diverticulum) and caecum. The samples were rinsed with phosphate buffered saline (PBS), conserved in the RNA stabilization Reagent “RNA-later” (Qiagen) and stored at room temperature for approximately 24 hours. Afterwards they were kept at -80°C until further use.

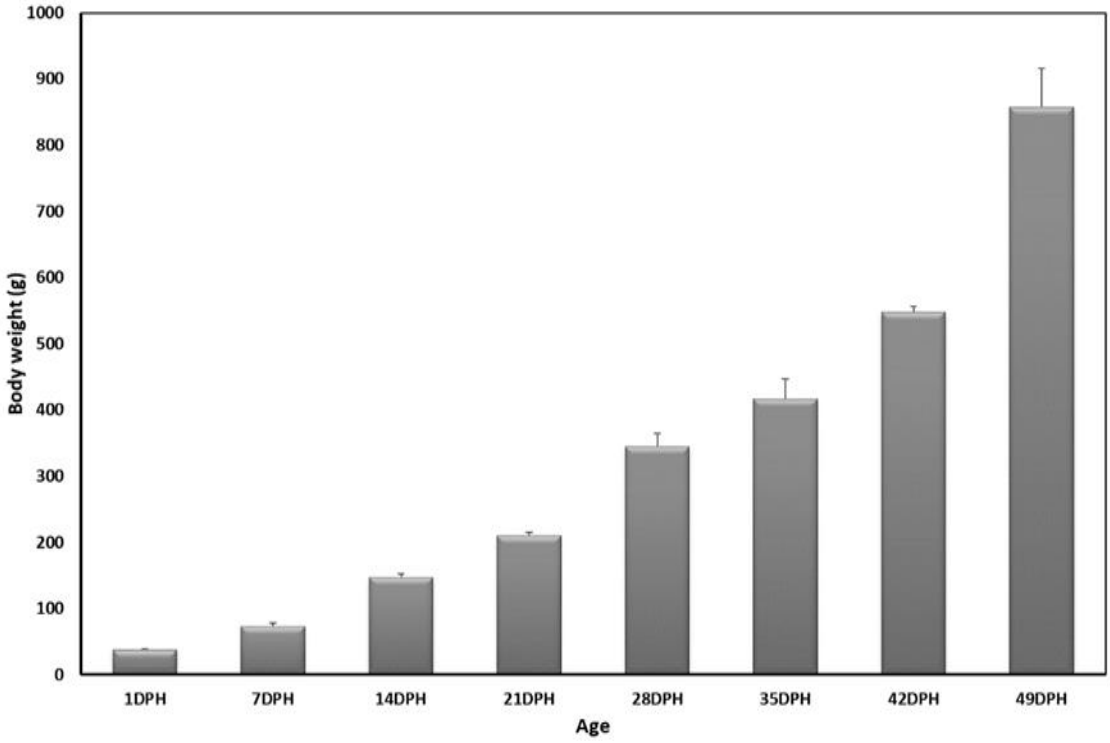

Figure 1. Body weight of the sampled birds. Results are presented as mean values and standard error of mean (SEM).

3.2. Sample preparation

After thawing, approximately 30 mg of tissue samples were collected in a 2 ml Eppendorf tube containing a steel bead (r = 5 mm) and 600 µl of “RLT plus®”-Buffer (Qiagen). The content was homogenized twice using a homogenizer (Tissue Lyzer®, Qiagen, Hilden, Germany) for 2 min at 30 Hz. Immediately afterwards the samples were centrifuged for 2 min at 13.000 rpm to dissolve the build-up foam.

3.3. RNA extraction

After the centrifugation, total RNA was extracted from the buffer-tissue suspension using the RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. To eliminate DNA contamination, the extracted RNA underwent a clean-up using DNaseI (Qiagen).

Purity and concentration of RNA were measured by 260nm/280nm and 230nm/260nm photometric extinction ratio using “Nanodrop2000” spectrophotometer and software (Thermo Fisher Scientific, Vienna, Austria). 260nm/280nm ratios between 1.5 and 2.3, concentrations above 150 ng/µl and nucleic acid purity equal or above 2 were accepted. Following the manufacturer's instructions, after dilution of the samples with RNase-free water to 300 ng/µl, samples were evaluated for their RNA integrity using the “RNA 6000 Nanokit” (Agilent Technologies) in the “Bioanalyzer_2100” automated electrophoresis instrument and software (Agilent Technologies, Waldbronn, Germany). RNA Integrity Number (RIN) was determined for all samples and it ranged from 8.5 to 10, indicating high RNA integrity.

All samples that could not meet those requirements were rejected and newly extracted. For further use in RT-qPCR the samples were diluted with RNase free water to 50 ng/µl and stored at -80°C.

3.4. RT-qPCR

One step Real time quantitative polymerase chain reaction (RT-qPCR) was performed by using “Brilliant III Ultra-Fast QRT-PCR Master Mix” kit (Agilent Technologies), “AriaMX real-time PCR system” (Agilent Technologies) and “Agilent AriaMX 1.7” software (Agilent Technologies). The primer and probes targeting the different tight junction proteins were used according to previous standardisation protocols (von BUCHHOLZ et al., 2021).

To normalize the gene of interest expression, the reference genes “60S ribosomal protein L13 (RPL 13)” and “TATA-box binding protein (TBP)” were used as described by MITRA et al. (2016). All samples were tested in duplicate.

The expression of CLDN2, CLDN10, CLDN19 and OCLN was determined via singleplex RT-qPCR, the expression of CLDN1, CLDN5, ZO1 and MD2, CLDN3 and CLDN7 was determined via multiplex RT-qPCR, respectively. To detect a possible DNA contamination, each sample was ran in duplex without the adding of reverse transciptase for each gene of interest. Additionally, for each plate, two wells as non template control (NTC) were included.

The thermal cycle profile for RT-qPCR was customized as follows: the reverse transcription phase for 10 min at 50°C followed by the hot start phase at 95°C for 3 min and 40 cycles of amplification at 95°C for 5 s and 60°C for 30 s.

The following formula was used for Cq normalization: $Cq + (N´q - C´q) \times S/S´$, where “N´q” is the mean Cq for the reference genes’ in all samples, “C´q” is the mean Cq for the reference genes in the sample, “S” is the average slope of the regressions of the standard plot for the genes of interest, and S´ is the average slope of the regressions of the standard plots for the reference genes as decribed by von BUCHHOLZ et al. (2021).

3.5. Statistical analysis

Statistical analyses were performed using IBM SPSS statistics 24®, SPSS software (Chicago, IL, USA). Results are presented as means with standard error of the mean (SEM). Data were tested for normality using the Kolmogorov-Smirnov’s test. TJ gene expression in different segments was compared by one-way ANOVA followed by a post hoc Duncan’s multiple range test. Differences were considered significant at a level of $P \leq 0.05$.

4. Results

4.1. Changes in tight junction gene expression

The mRNA gene expression of the established nine TJ proteins (CLDN1, CLDN3, CLDN5, CLDN7, CLDN10, CLDN19, OCLN, MD2 and ZO1) was measured in in the intestinal epithelia of jejunum and caecum of SPF layer chicken. For normalisation, reference housekeeping genes RPL13 and TBP were run in a duplex PCR.

4.1.1. Barrier-forming tight junction proteins

Significant changes in mRNA expression of barrier-forming claudins were observed in both jejunum and caecum (Figure 2). In jejunum, relative gene expression of CLDN1 was significantly decreased ($P \leq 0.001$) from 7-49 days post hatching (DPH) compared to the 1 DPH. CLDN3 was significantly decreased ($P \leq 0.001$) at 28, 35 and 42 DPH as compared to the 1 DPH and increased again thereafter. However, the relative gene expression of CLDN5 only increased at 35 DPH and decreased again thereafter.

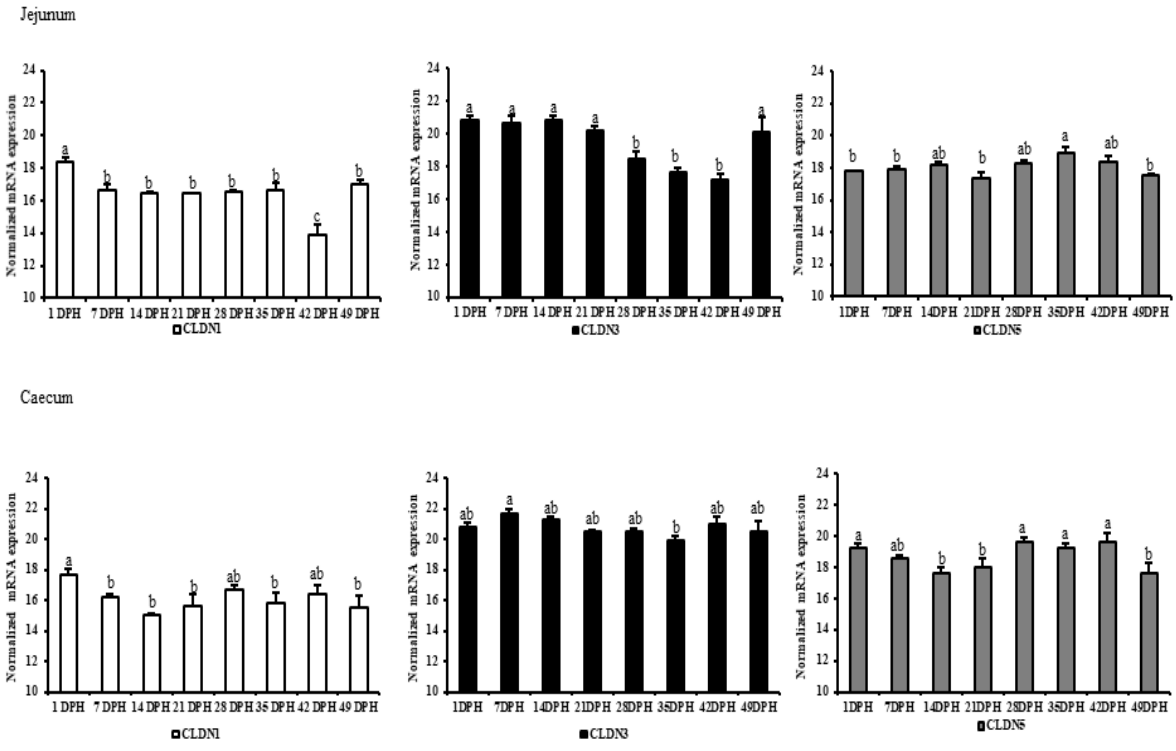

Figure 2. Developmental changes in the mRNA expression of the three barrier-forming claudins in jejunum and caecum of SPF layers. Results are presented as means with SEM (n = 5 birds), means with different superscripts are significantly different ($P < 0.05$) (ANOVA and Duncan's test).

Similarly, in caecum, CLDN1 showed a significant decrease in the gene expression at all time points compared to the 1 DPH. Expression of CLDN3 was significantly decreased ($P \leq 0.001$) at 35 DPH compared to the other time points. However, the relative gene expression of CLDN5 was not constant, increased at 35- 42 DPH and decreased again thereafter.

4.1.2. Pore-forming tight junction proteins and tight junction proteins with ambiguous functions

CLDN7, CLDN10 and CLDN19 showed a very heterogenous pattern in mRNA expression throughout the experimental time in the intestinal epithelia of both jejunum and caecum (Figure 3).

The relative gene expression of CLDN19 was significantly increased at 35 and 42 DPH compared to the other time points in jejunum. CLDN10 showed an ambiguous expression with three notable minima on 21, 35 and 42 DPH. CLDN7 expression was higher from 1 DPH to 21 DPH and significantly decreased ($P < 0.05$) from 28 DPH onwards.

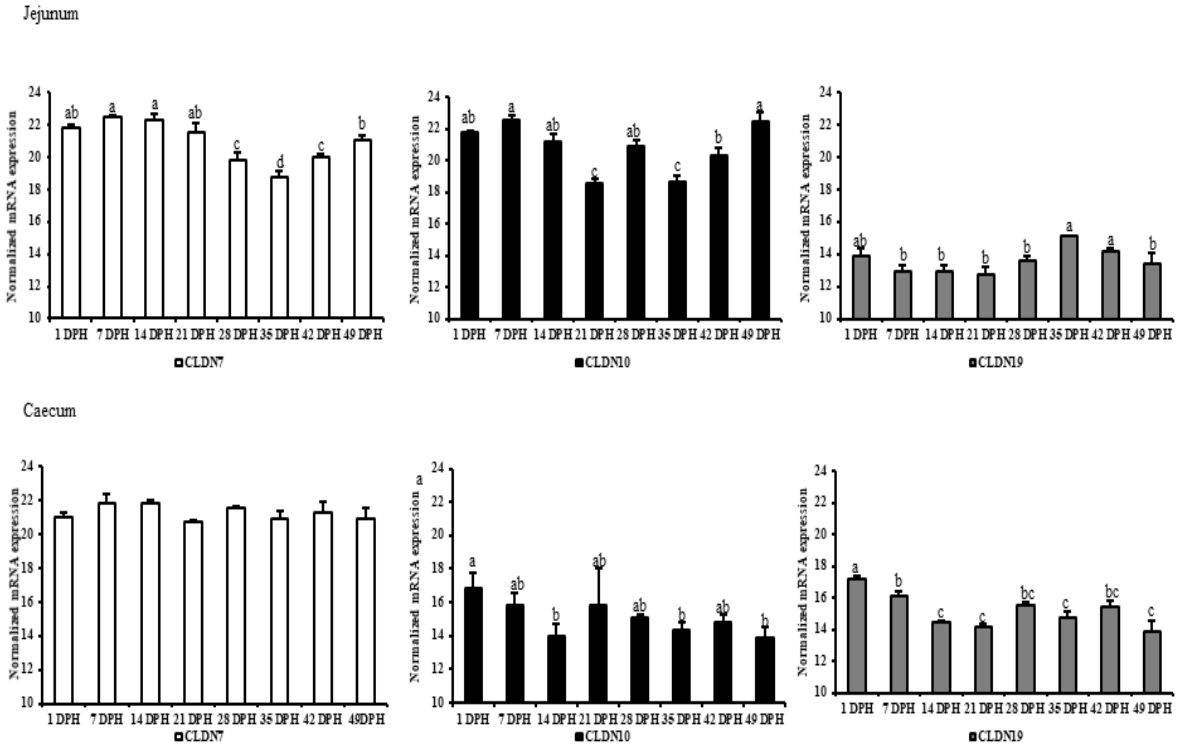

Figure 3. Developmental changes in the mRNA expression of the three pore-forming or ambiguous claudins in jejunum and caecum of SPF layers. Results are presented as means with SEM (n = 5 birds), means with different superscripts are significantly different ($P < 0.05$) (ANOVA and Duncan's test).

In caecum, relative mRNA expression of CLDN10 and CLDN19 was significantly ($P < 0.05$) higher at 1 DPH and decreased from 7 DPH onwards. In contrast, CLDN7 was expressed at a constant level throughout the trial.

4.1.3. Cytosolic and tight junction-associated MARVEL proteins (TAMP)

Overall the studied TAMP showed a nearly constant mRNA expression pattern over the experimental period (Figure 4).

OCLN showed a very constant expression in the intestinal epithelia of both jejunum and caecum with a significant ($P < 0.05$) increase in mRNA expression in jejunum at 14-21 DPH and in caecum at 7 DPH and 49 DPH.

The mRNA expression of MD2 in jejunum increased significantly ($P < 0.05$) at 14 DPH, and decreased thereafter then increasing again at the end of the trial (49 DPH). In caecum, the MD2 expression increased only at 7 DPH and plateaued for the whole observed timeframe.

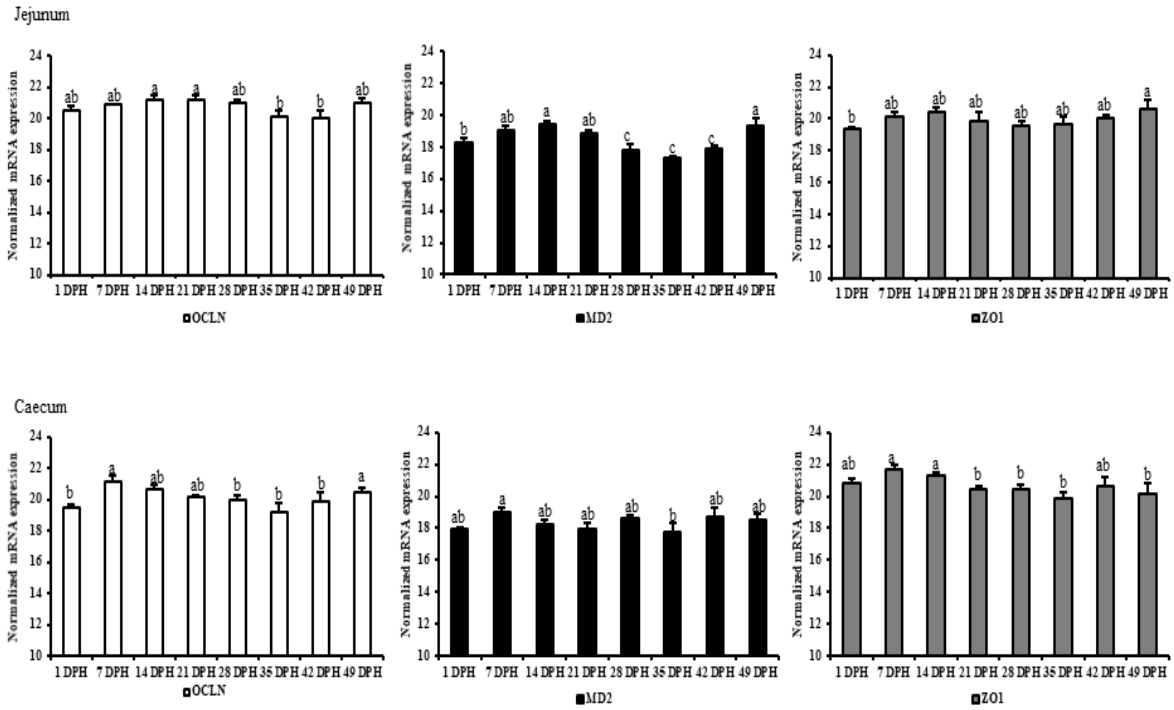

Figure 4. Developmental changes in the mRNA expression of the OCLN, MD2 and ZO1 in jejunum and caecum of SPF layers.

Results are presented as means with SEM (n = 5 birds), means with different superscripts are significantly different ($P < 0.05$) (ANOVA and Duncan's test).

The mRNA expression of ZO1 was stabilized in jejunum from 1 DPH to 42 DPH followed by an increase in expression at 49 DPH. However, in caecum, the expression of ZO1 increased only at 7-14 DPH and decreased significantly ($P < 0.05$) thereafter.

5. Discussion

Tight junctions (TJ) play a major role in upholding an intact intestinal barrier (VAN ITALLIE et al., 2004; OSSELAERE et al., 2013; SAITOH et al., 2015; LEE et al., 2018; TSUKITA et al., 2019). They create a barrier to the luminal microorganisms and their secreted products as well as free diffusion of fluids, electrolytes and macromolecules (VAN ITALLIE et al., 2004). Consequently, TJ play an important role in the physiological function of epithelial cells. Some microorganisms alter intestinal barrier function by disruption of tight junctions and initiation of inflammatory cascades e.g. *Clostridium perfringens* type A (SAITOH et al., 2015; EMAMI et al., 2019). Generally, a disruption of gut barrier function is a common characteristic of many local and systemic infections, and a leaky gut contributes to the severity of clinical symptoms.

Many studies have demonstrated the role of TJ proteins in the intestinal barrier formation of mammals; however, the organization of the paracellular barrier of the chicken intestine is still a matter of debate. There is limited data on tight junctions in chickens, obtained mainly from broilers (COLLINS et al., 2013; von BUCHHOLZ et al., 2021; OSSELAERE et al., 2013; AGUIRRE et al., 2021), which grow much faster than layers and consequently have different metabolic requirements. No information is available on the developmental changes in the expression of tight junction proteins in the intestine of layers. Therefore, the aim of this study was to investigate the developmental changes in the TJs expression in layers.

The present study revealed significant differences in the expression of cytosolic mRNA coding for barrier-forming TJ proteins in both jejunal and ceacal epithelia. The decrease in the expression of barrier-forming CLDN1 and CLDN5 in jejunum and caecum may indicate a reduction of epithelial barrier integrity during that period (FURUSE et al., 2002; GÜNZEL et al., 2013; CHELAKKOT et al., 2018). In contrast to broilers, laying hens showed a very heterogenous pattern in TJ mRNA expression and did not reach a plateau until the end of the experiment (von BUCHHOLZ et al., 2021; AGUIRRE et al., 2021). In broilers, the TJ mRNA remained constant from the age of 21 days until slaughter (35 days), which can be explained by the faster development of the intestinal tract of broilers (von BUCHHOLZ et al., 2021).

CLDN7-gene expression was higher in jejunum at the first three weeks of age and decreased thereafter. CLDN7 has ambiguous functions, one of which is to increase paracellular conductance of Na^+ ions (ALEXANDRE et al., 2005). MITJANS et al. (1997) reported that the increase in structural TJ proteins together with increased ion

permeation across the paracellular space appears to coincide with cell proliferation and is therefore crucial for the functional development of the small intestine. In caecum, most of the investigated TJ genes showed changes in mRNA expression after hatch with the noticeable exception in the pore-forming claudins CLDN7. In chickens, the main function of the caecum is microbial digestion. It was reported that the caecal microbiota reaches a mass of 10^{11} per gram digesta at the third day after hatching in broilers with no significant fluctuations for the next 30 days of life (APAJALAHTI et al., 2004). Broilers and layers have different metabolism, very different lifespans in normal commercial production, and have different dietary requirements. Therefore, the composition of the gut microbiota in these two lines is different. The microbiota differences can differentially influence the intestinal mRNA expression (PANDIT et al., 2018).

In addition, the quantification of cytosolic mRNA expression alone, as conducted in the current study, may not allow a direct conclusion regarding protein activity, as phosphorylation and posttranslational modification play an important role in TJ-activity as well (VAN ITALLIE et al., 2018; PROSKOWIEC-WEGLARZ et al., 2020). This is supported by the fact that CLDN half-life varies between proteins (VAN ITALLIE et al., 2019), so high mRNA expression may be a sign of higher protein turnover rather than high expression of TJ. Therefore, the characterization of TJ at the protein level remains to be resolved.

6. Conclusion

The results of the current study showed that the expression of TJ in layers, in contrast to broiler chickens, followed a less stringent pattern of decreases and increases in the first seven weeks of life. Instead, it showed a kind of undulating pattern of mRNA expression of all TJs in both jejunum and caecum except CLDN5 and CLDN19. This can be explained by the faster development of the intestinal tract of broilers in comparison to layers, indicating a breed-specific influences on the developemental profile of tight junctions mRNA expression.

Since TJ plays a central role in the intestinal barrier, knowledge of the physiological expression of TJ proteins is of great interest to understand physiological and pathophysiological changes in the chicken intestine. Ultimately, the constitution of TJs can be used as a marker for gut integrity. Furthermore, age-associated changes of mRNA expression in the layers intestinal TJ barrier could help to demonstrate how compartmental separation and transepithelial transport take place at different ages. Simultaneously, it points to differential regulation in paracellular ion permeability in layers and broilers after hatch. However, the localization and quantification of those TJ proteins in layers when they are in production needs further investigation.

7. Summary

Tight junctions (TJ) are essential for maintaining physiological homeostasis and structural integrity in epithelial and endothelial tissues. This is extremely important in the gut, as it constitutes an initial organ and huge surface exposed to external biological and chemical agents influencing bird's health and serves as the first line of defense between the host and the luminal environment.

In the present study, a developmental profile of tight junction proteins mRNA expression in the jejunum and caecum of SPF layer type chickens during the first seven weeks of life was investigated (n = 40). For this purpose, tissue samples from freshly euthanized birds were collected at the age of 1-49 days. The mRNA expression of the six claudins (CLDN) CLDN1, CLDN3, CLDN5, CLDN7, CLDN 10, and CLDN 19 and the three tight junction associated MARVEL proteins (TAMP) Occludin (OCLDN), MARVELD2 (MD2) and zonula occludens 1 (ZO1) were quantified by RT-qPCR.

In the jejunal epithelia, relative gene expression of CLDN1 was significantly decreased ($P \leq 0.001$) from 7-49 days post hatching (DPH) compared to the 1 DPH. CLDN3 expression significantly decreased ($P \leq 0.001$) at 28, 35 and 42 DPH as compared to the 1 DPH and increased again at 49 DPH. However, the relative gene expression of CLDN5 was only increased at 35 DPH and then decreased again. Furthermore, in caecum, CLDN1 showed a significant decrease in the gene expression at all time points compared to the 1 DPH. CLDN3 expression significantly decreased ($P \leq 0.001$) at 35 DPH compared to the other time points. However, the relative gene expression of CLDN5 was not constant, increased at 28-42 DPH and then decreased again.

Similarly, the pore-forming and cytosolic TJ showed a very heterogenous pattern in mRNA expression throughout the experimental period in the intestinal epithelia of both jejunum and caecum. The results revealed that the expression of TJ proteins in laying hens, in contrast to broilers, generally followed a less strict pattern with a significant decrease and increase over the experimental period.

Overall, the determination of the segment-specific changes in the expression of TJ mRNA expression can provide a deeper understanding of the molecular mechanisms underlying the pathophysiological changes in the gut of layers with different physiological and pathological stimuli. Therefore, an increased knowledge of the molecular composition of tight junction proteins in chickens is important to understand certain pathogenic pathways, as the composition of tight junctions can be used as a marker for gut integrity.

8. Zusammenfassung

Die vorliegende Arbeit hatte zum Ziel, die Genexpressionsprofile für Tight-Junction-Proteine in Jejunum und Caecum von Legehühnern (n = 40) in den ersten sieben Lebenswochen (Kükenphase) zu erstellen.

Tight Junctions spielen eine zentrale Rolle in der lebenswichtigen Erhaltung einer physiologischen Homöostase und strukturellen Integrität in Epithelien und Endothelien aller Wirbeltiere (und fast aller Chordatiere). Sie verbinden benachbarte Zellen, um eine semipermeable Membran zu schaffen, eine Grundlage für komplexes Leben. Insbesondere im Darm, der eine im Vergleich zum Körpervolumen enorme Oberfläche zur Außenwelt hin darstellt, ist diese Funktion essenziell.

Mittels RT-qPCR wurde mRNA der sechs Claudine (CLDN) CLDN1, CLDN 3, CLDN5, CLDN7, CLDN10 und CLDN19 sowie die der drei Tight-Junction-assoziierten-MARVEL-Proteine (TAMP) Occludin (OCLDN), MARVELD2 (MD2) und Zonula occludens 1 (ZO1) über einen Zeitraum von sieben Wochen detektiert, quantifiziert und anhand zweier konstant expremierter Kontrollgene (RPL13 und TBP) normalisiert. Die gewonnen Daten wurden kohortenübergreifend mittels ANOVA und Duncan-Test auf statistische Signifikanz überprüft. Vergleichbare Arbeiten waren vorher zur Embryonalentwicklung des Huhnes und zur Genexpression im Darm heranwachsender Masthühner (Broiler) publiziert worden, nie jedoch für Legehybriden.

Im Jejunum nahm die Expression von CLDN1 über die gesamte Beobachtungsperiode hinweg, bezogen auf das Expressionsniveau am Schlupftag, signifikant ($P \leq 0.001$) ab. CLDN3 wurde ebenfalls am 28., 35., und 42. Lebenstag signifikant ($P \leq 0.001$) schwächer exprimiert als am Schlupftag, am 49. Lebenstag allerdings wieder stärker. Die Expression von CLDN5 nahm nur am 35 Lebenstag zu und nachfolgend wieder ab. Auch im Caecum nahm die Expression von CLDN1 vom Niveau des Schlupftages betrachtet stetig ab, während die Expression von CLDN3 am 35. Lebenstag ein Minimum erreichte. Die Expression von CLDN5 zeigte keine konstante Tendenz sondern ein Maximum bei 28-42 Lebenstagen und sank anschließend wieder. Ein ähnliches Bild zeigten jene untersuchten TJ, die zur Gruppe der porenbildenden und der strukturgebenden TJ gehören: Ihre Expression zeigte ein heterogenes Bild über den beobachteten Zeitraum sowohl im Jejunum als auch im Caecum.

Dieses Ergebnis zeigt, dass die Expression von TJ in Legehybriden in den ersten Lebenswochen ein weniger offensichtliches Muster mit signifikanten Zu- und

Abnahmen beschreibt als bei Masthybriden, was angesichts der unterschiedlichen Wachstumsraten der beiden Linien erklärbar ist und zu einem gewissen Grad erwartbar war.

Zusammengefasst kann eine genaue Kenntnis über die Expression relevanter TJ abhängig von Alter und Darmabschnitt ein besseres Verständnis der molekularen Mechanismen bedingen, die zu relevanten pathophysiologischen Veränderungen im Darm von Legehybriden führen.

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