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Effects of natural endocrine active substances on tight junction expression in the intestinal epithelium modeled with Caco-2/HT29-MTX-E12 co-culture

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Abstract English

Impaired intestinal barrier integrity is a relevant etiologic factor for the development of various diseases including inflammatory bowel diseases (IBD) and colon carcinoma in humans. Under healthy conditions, the intestinal barrier effectively regulates the passage of nutrients, water and protects against harmful substances potentially present in the diet. To ensure these functions, intestinal epithelial cells are strongly connected via specialized proteins, the so-called tight junctions. Tight junctions do not only link epithelium cells but also regulate paracellular transport of dietary compounds and therefore have a strong impact on intestinal barrier function. Research suggests that various dietary substances might influence barrier integrity in the intestine by modifying the expression of integral membrane constituents including tight junction proteins. Recent studies indicate that endocrine active chemicals (EAC) or endocrine disruptive chemicals (EDC) might influence the expression of claudin 4 (CLDN4) and other membrane proteins. This study focus specifically on the effects of selected natural EACs on the tight junction protein CLDN4. The natural EACs to be tested include phytoestrogens genistein (GEN), daidzein (DAI) and chrysin (CHRY), the mycoestrogen zearalenone (ZEN) and the endogenous substances 17β-estradiol (E2) and cholesterol (CHOL). Phytoestrogens are naturally occurring in plants and are commonly found in diet. Their structural similarity to 17β-estradiol induces estrogenic activity. Mycoestrogens are also endocrine-active and can be consumed via crops contaminated by fungi. The intestinal epithelium was modeled by a co-culture of Caco-2 and mucus producing HT29-MTX-E12 cells. The co-culture forms a cell monolayer with mucus during 7-day differentiation time. Mucus reduced conditions were also evaluated in the study to investigate effects brought about by diseases with reduced mucosal lining in the gut like irritable bowel syndrome. The cells were treated with the substances of interest for 24 h, followed by immunostaining of CLDN4. Imaging with confocal microscopy (63X) enabled detailed localization and evaluation of the tight junction protein. Interestingly, the compounds CHRY, CHOL and E2 significantly impacted CLDN4 expression in this model. Additionally, the effect of the test substances on membrane fluidity was investigated due to the possible interaction of membrane composition and membrane protein function. Another non-endocrine natural contaminant, the mycotoxin deoxynivalenol (DON), was included as reference compound to complement the observations by EACs, also with potential effects on barrier integrity. Incubation with DON increased CLDN4 expression in the 7-day differentiated model.

Abstract Deutsch

Eine unzureichende Barrierefunktion des Darmtraktes ist ein relevanter ätiologischer Faktor für die Entstehung verschiedener Krankheiten wie entzündliche Darmerkrankungen und Darmkrebs im Menschen. Unter physiologischen Bedingungen reguliert die Darmbarriere effektiv den Transport von Nährstoffen und Wasser sowie schützt vor potenziell schädlichen in der Nahrung vorkommenden Substanzen. Intestinale Epithelzellen sind durch spezialisierte Proteine stark miteinander verbunden, den sogenannten Tight Junctions. Tight Junctions verbinden nicht nur Epithelzellen miteinander, sondern regulieren auch den parazellulären Transport von Nahrungsbestandteilen und haben daher einen starken Einfluss auf die Barrierefunktion des Darms. Die Forschung legt nahe, dass verschiedene Substanzen in der Nahrung die Barrierefunktion des Darms verändern können, indem sie die Expression integraler Membranbestandteile so wie Tight Junction Proteine beeinflussen. Aktuelle Studien weisen darauf hin, dass endokrin-aktive Chemikalien (EAC) oder endokrine Disruptoren (EDC) die Expression von claudin 4 (CLDN4) und anderen Membranproteinen verändern können. Die hier vorliegende Masterarbeit beleuchtet den Effekt ausgewählter natürlich vorkommender EACs auf das Tight Junction Protein CLDN4. Die getesteten natürlichen EACs beinhalten die Phytoöstrogene Genistein (GEN), Daidzein (DAI), Chrysin (CHRY), das Mykoöstrogen Zearalenon (ZEN) und die endogenen Substanzen 17β-Estradiol (E2) und Cholesterol (CHOL). Phytoöstrogene kommen natürlicherweise in Pflanzen vor und sind häufige Bestandteile der Nahrung. Durch ihre strukturelle Ähnlichkeit zu 17β-Estradiol verfügen sie über östrogene Aktivität. Mykoöstrogene sind auch endokrin-aktiv und werden über pilzbefallenes Getreide konsumiert. Das Darmepithel wurde durch ein Kokultur aus Caco-2 und Mukus produzierenden HT29-MTX-E12 Zellen modelliert. Nach 7 Tagen Differenzierungszeit, bildet die Kokultur eine einschichtige Zelllage mit Mukus. Um Effekte abzuschätzen, die bei Erkrankungen mit geschädigter Schleimhaut im Darm, wie dem Reizdarmsyndrom, auftreten, wurden auch Untersuchungen mit reduziertem Mukus durchgeführt. Nach 24-stündiger Inkubation der Kokultur mit den gewählten Substanzen wurde CDLN4 mit Antikörper markiert. Konfokal Mikroskopie (63X) ermöglichte eine detaillierte Bestimmung des CLDN4 Proteins. Interessanterweise hatten die Substanzen CHRY, CHOL und E2 einen starken Effekt auf die CLDN4 Expression in dem hier verwendeten Modell. Weiters wurde der Einfluss der Testsubstanzen auf die Membranfluidität untersucht, da ein möglicher Zusammenhang zwischen Membranzusammensetzung und Funktion der Membranproteine besteht. Zusätzlich wurde Deoxynivalenol (DON), eine weitere Substanz mit potenziellen Auswirkungen auf die Barrierefunktion, als Referenzsubstanz untersucht, um die Beobachtungen mit einer nicht endokrin-aktiven natürlichen Kontaminante zu untermauern. Inkubation mit DON führte zu einer erhöhten CLDN4 Expression in der 7-Tage differenzierten Kokultur.

Acknowlegdements

Ein besonderer Dank gilt meiner Betreuerin Giorgia Del Favero, für die Möglichkeit meine Masterarbeit durchzuführen, sowie für ihre stetige Unterstützung und fachliche Betreuung. Ich möchte auch der gesamten Arbeitsgruppe, insbesondere Janice Bergen, für die Unterstützung in dieser spannenden, lehrreichen Zeit danken. Ein aufrichtiger Dank gilt Prof. Doris Marko für die zur Bereitstellung der Einrichtungen und Materialien des Departments für Lebensmittelchemie und -toxikologie. Teile der Daten dieser Masterarbeit sind Teil des Manuskripts "Insights on the potential of endocrine active chemicals to modulate intestinal barrier function: a combined in vitro/in silico approach delineates tight junction protein claudin-4 as a target", Bergen et al. 2026, welches sich zurzeit in der Begutachtungsphase befindet.

Abbreviations

bw: body weight

CHOL: Cholesterol

CHRY: Chrysin

CLDN4: Claudin 4

CTB: CellTiter Blue®

DAI: Daidzein

DON: Deoxynivalenol

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethylsulfoxide

EACs: Endocrine active chemicals

EDCs: Endocrine disruptive chemicals

ER: Estrogen receptor

ERα: Estrogen receptor alpha

ERβ: Estrogen receptor beta

E2: 17-beta-Estradiol

FBS: Fetal bovine serum

GEN: Genistein

ICE: Integrated Chemical Environment

NAMs: New approach methodologies

NSAIDS: non-steroidal anti-inflammatory drugs

PBPK: Physiological based pharmacokinetic

PBS: Phosphate buffered saline

PBS-A: Phosphate buffered saline (variation A)

TEER: Transepithelial electrical resistance

TJ: Tight junction

ZEN: Zearalenone

ZO-1: Zonula Occludens 1

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1 Introduction

1.1 Endocrine Activity and xenobiotics

The endocrine system is an important communication network in the human body that regulates the development and growth of organs and tissues, metabolism and many other physiological processes[1]. It works via secretion of hormones, transport through the blood stream and binding towards receptors on or in target cells[1]. Hormones are synthesized and secreted by various glands, such as the thyroid, the ovaries, the tests and the hypothalamus[1]. Less well known, the duodenum, the stomach, the skeletal muscle, the kidneys, the liver, the adipose tissue, the skin, and the heart can produce, secrete or interact with hormones as well[2]. Apart from physiological active molecules, xenobiotics with hormonal activity can also enter and influence the endocrine system[2,3]. Nowadays we are aware that several contaminants, pesticides and chemicals applied in consumer goods, medicine and pharmaceuticals may influence or interfere with the endocrine activity in the body. Furthermore, natural products contain so-called endocrine active substances (EAC), e.g. phytoestrogens. The human body reacts to the exposure with EACs and changes on the endocrine system via various homeostatic mechanisms, but not without limit[2]. If the body cannot adapt to certain EACs, they lead to adverse effects and are classified as Endocrine disruptive substances (EDC) as a consequence. One major route of exposure for EDCs is oral consumption. EDCs can reach the diet as residues from pesticides and biocides, plasticizers in food packaging, persistent environmental contaminants, substances produced during food processing, plant-derived natural compounds and mycotoxins from fungi[2].

1.2 Intestinal Epithelium

Among human organs, the intestine has the greatest total surface area in contact with the external environment, followed by skin and pulmonary tissue[4]. Next to the uptake of nutrients, the intestine acts as barrier against pathogens, toxins, antigens and other ingested compounds[4]. A key role in building up a physical barrier in the gut are intestinal epithelial cells[4]. Epithelial cell layers generally consist of five specialized cell subtypes such as enteroendocrine cells, paneth cells, microfold cells, enterocytes and goblet cells[5]. For example, enteroendocrine cells contribute to the digestion of nutrients via secretion of peptide hormones when stimulated via luminal constituents[6]. Paneth cells have a defense function via production of anti-microbial peptides while microfold cells serve as protection against antigens microorganisms via phagocytosis and transcytosis[7]. Enterocytes are the most prevalent cells in the

small intestine and form the main unit of the physical intestinal barrier[4,7]. A functional intestinal barrier strictly regulates the passage of ingested substances supported by specialized protein structures such as tight junctions, adherens junctions and desmosomes[4]. In addition, intestinal epithelium cells also contribute to the chemical barrier in the gut via synthesis of mucins and antimicrobial peptides[4]. Hereby, the goblet cells produce mucins which either coat the epithelial cell layer as a gel-like mucus or stay cell surface-associated as the so-called glycocalyx[5]. The composition and properties of the mucus are also potentially influenced by the gut microbiome[8]. The microbiome is an assembly of microorganisms in the gut forming a symbiotic relationship with their human host[9]. The gut microbiome does not only contribute to the mucus layer[9] but can also modulate the expression and localization of tight junction proteins[10]. Furthermore, the microbiome in the gastrointestinal tract possibly metabolizes ingested substances including xenobiotics[9]. Vice versa, xenobiotics are also shown to influence the activity and structure of the microbiome, adding another layer of complexity to the system[9].

To enable efficient absorption of nutrients and fast regeneration, the small intestine contains numerous villi structures and crypts[4]. Originating from the crypts, cells migrate towards the villi tip while proliferating and differentiating and are finally extruded in the intestinal lumen after 4-5 days[11]. The fast life cycle leads to the shedding of multiple, approximately 10^{10}, cells each day[11]. Continuous cell shedding in the intestine demands a redistribution of tight junction proteins to prevent breaches in the epithelium and to preserve intestinal barrier integrity[7].

1.3 Barrier function in gut health

Insufficient barrier function through disruption of the intestinal barrier has shown adverse effects on human health, including chronic inflammatory disorders in and outside of the intestine[4]. A disrupted intestinal barrier facilitates the entry of microorganisms and antigens, causing proinflammatory activation of mucosal immune cells[12]. Increased probability for the development of inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease as well as colon carcinoma are possible consequences[12]. Further, recent studies suggest that insufficient barrier function may promote pathogenesis of non-gut related diseases, such as Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, and metabolic dysfunction associated steatohepatitis[4]. This indicates the importance of the intestinal barrier function for human health. A loss in barrier integrity in the epithelium can derive from epithelial injury, alterations in tight junction function[13] and even changes in the intestinal microbiota[10]. Initiative factors for insufficient barrier function are found in diet and lifestyle, such as food, medication including non-steroidal anti-inflammatory drugs (NSAIDS), stress, smoking and alcohol[5]. Addressing the latter, several *in vivo* and *in vitro* studies indicate the adverse effects of ethanol

and its metabolite acetaldehyde on the epithelial barrier integrity[14]. In the diet, substances with positive and negative effects on the cell layer permeability have been reported, opening up a research field of great importance for the aforementioned reasons[5].

1.4 Aim of the Study

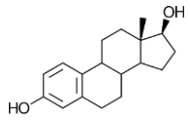
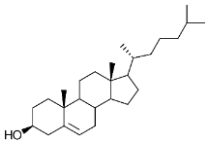
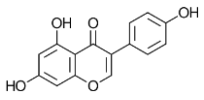
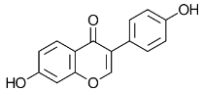
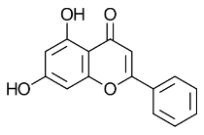
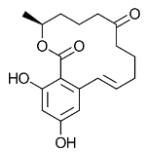
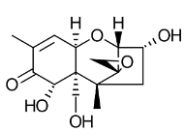
Absorption of nutrients and water is the most prominent function of the human intestine. Additionally, the intestine serves as crucial barrier against potentially harmful substances present in the diet such as pathogens, antigens or toxins. The barrier function in the epithelium, particularly the paracellular permeability, is highly dependent on protein complexes, such as tight junction proteins[4]. However, novel research has indicated the potential of foodborne substances in changing the expression of the specialized protein structures and thereby permeability. In 2022, research conducted by Groestlinger J et al.[15] observed alterations in the localization of integral membrane proteins after exposure to *Alternaria* mycotoxins in human colon epithelial cells. Incubation with alternariol (AOH) and altertoxin-II (ATX-2) changed expression of occludin (Ocln), zona occludens 1 (ZO-1) and claudin 4 (CLDN4), which play key roles for intestinal barrier integrity. Furthermore, endocrine activity of *Alternaria* mycotoxins *in vivo* was reported by Crudo F et al. 2022.[16] Not only AOH, but also other endocrine active substances such as genistein (GEN), bisphenol A (BPA) and α-zearalenol (α-ZEL) do affect motility and adhesion in breast cancer cells, according to *in vitro* studies by Del Favero G et al., 2024.[17] In the cited study, the actin cytoskeleton and integrin β1 expression in cells were modified by short-term exposure with tested dietary xenoestrogens. These findings suggest an effect of endocrine active substances on the expression/function of proteins supporting cell adhesion, which could potentially provide a mechanistic link between dietary exposures to EACs and the (de-)regulation of gut homeostasis.

The aim of the present study was to evaluate the effect of natural endocrine active substances on tight junction expression and barrier function in the intestinal epithelium. This was conducted by combining biochemical toxicological analyses with immunofluorescence studies via confocal microscopy. Human colon cancer cells Caco-2/HT29-MTX-12 mimicking the intestinal epithelium were incubated with the test substances at concentrations that can be realistically expected in the gut. The phytoestrogens GEN, daidzein (DAI) and chrysin (CHRY) and the mycoestrogen zearalenone (ZEN) were the focus of investigation. Due to possible interactions of membrane composition and fluidity with membrane proteins[18,19], the effects of the test compounds on the membrane fluidity were additionally investigated.

2 Theoretical Background

2.1 Endocrine active and disruptive substances

In this study, the endogenous substances 17β-estradiol (E2) and cholesterol (CHOL), the phytoestrogens GEN, DAI and CHRY, the mycoestrogen ZEN as well as the non-estrogenic mycotoxin deoxynivalenol (DON) were tested. A summary of the compounds including chemical structure, main sources/occurrence, average estimated exposure and current regulations is given in **Tab 2.1**.

SUBSTANCE	STRUCTURE	SOURCES	ESTIMATED EXPOSURE
17β-ESTRADIOL		**Endogenous**[20]	**NOEL:** 5 µg/kg bw per day[21] **ADI:** 50 ng/kg bw = 3.5 µg/day (70 kg)[21] **Estim. conc. in small intestine (based on ADI):** 33.3 µg/L[22]
CHOLESTEROL		**Endogenous, dietary lipids**[23]	**Average daily intake for adults:** 200 - 550 mg per day = 2.86 - 7.86 mg/kg bw per day (70 kg)[24] **Estim. conc. in small intestine:** 1.90-5.24 g/L[22]
GENISTEIN **Isoflavone**		**Soy, soy products**[25]	**Average daily intake for adults:** 0.2 mg/kg bw per day = 14 mg/day (70 kg)[26] **Estim. conc. in small intestine:** 133 mg/L[22]
DAIDZEIN **Isoflavone**		**Soy, soy products**[27]	**Average daily intake for adults:** 0.2 mg/kg bw /day = 14 mg/day (70 kg)[26] **Estim. conc. in small intestine:** 133 mg/L[22]
CHRYSIN **Flavone**		**Honey, propolis**[28]	**Average daily intake for adults:** 70.6 µg/day = 1 µg/kg bw per day (70 kg)[28,29] **Estim. conc. in small intestine:** 0.672 mg/L[22]
ZEARALENONE **Mycoestrogen**		**Cereals**[2]	**Average daily intake for adults:** 2.4 - 29 ng/kg bw per day = 0.17 - 2.03 µg/day (70 kg)[30] **TDI**: 0.25 µg/kg bw per day = 17.5 µg/day[30] **Estim. conc. in small intestine (based on TDI):** 166.7 µg/L[22]
DEOXYNIVALENOL **Mycotoxin**		**Cereals**[31]	**Average daily intake for adults:** 0.22- 0.58 µg/kg b.w. per day[31] **TDI**: 1µg/kg bw per day = 70 µg/day (70kg)[31] **Estim. conc. in small intestine (based on TDI):** 666.6 µg/L = 2.25 µM[22]

Tab 2.1 summary: structure, sources, exposure & regulations of 17β-estradiol, cholesterol, genistein, daidzein, chrysin, zearalenone, deoxynivalenol.

2.2 Endogenous compounds

The human gut produces more than 20 different hormones and hereby contributes to the regulation of several physiological processes[32]. Responsible for hormone production in the gut are enteroendocrine cells, which secret hormones that contribute to systemic and local homeostasis[33]. Upon stimulation with luminal or neuronal factors, enteroendocrine cells release various hormones such as glucose-dependent insulinotropic polypeptide (GIP), cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1)[33]. Gut-derived hormones fulfill a crucial function in the regulation of nutrient absorption, appetite and mobilization of fat stores and thus are essential for energy balancing[32]. Further, glucose homeostasis is supported by hormonal secretion in the gut following receptor activation in pancreas and liver[32]. Novel research suggests, that intestinal epithelium cells even contribute to intestinal immune homeostasis via secretion of glucocorticoids, which are steroid hormones synthesized from cholesterol[34,35].

2.2.117β-estradiol

17β-estradiol (E2) is an endogenous steroid hormone with a hydroxyl group at the 17th carbon in β-configuration and it binds to the estrogen receptors (ER)[20]. ER are divided into two receptor types ERα and ERβ, which are located on different chromosomes (ERα in chromosome 6, ERβ on chromosome 14). The docking of E2 leads to specific expression patterns, dependent on the activated receptor[36]. The receptor ERα is crucial for the female reproduction system and present in ovaries, liver, central nervous system and breast tissue. In contrast, ERβ is important for the formation and development of the gastrointestinal tract, the urogenital tract and the cardiovascular system[37]. Through activation of ERα and ERβ, estradiol showed an inhibitory effect on the NF-κB receptor in various cell lines. The transcription factor NF-κB is substantial for the response to inflammation, autoimmune diseases, cancer, diabetes and various other pathological conditions[38].

To reduce postmenopausal symptoms, E2 is commonly used in hormone replacement therapy (HRT) via oral, transdermal or intramuscular administration[39]. The World Health Organization (WHO) Committee defined an Acceptable daily intake (ADI) for 17β-estradiol of 0-50 ng/kg bw per day. This is based on the No observed effect level (NOEL) of 5 µg/kg bw/day evaluated in studies with postmenopausal women[21]. Further, the ADI is equivalent to 3.5 µg/day (70 kg) which leads to an estimated concentration in the intestine of 33.3 µg/L, based on the average volume in the intestine under fasting conditions of 105 mL[22].

2.2.2 Cholesterol

Cholesterol (CHOL) is an endogenous lipid made of a rigid four ring system with one hydroxyl group and a hydrocarbon tail. In the human body, CHOL is relevant for the composition of membranes, membrane protein function, transmembrane signaling and intracellular transport[23]. Through steroidogenesis, CHOL is converted to steroid hormones, e.g. 17β-estradiol[40]. CHOL originates from two major sources: it is either synthesized *de novo* in cells from acetyl-CoA via the mevalonate pathway or obtained through the diet[23]. After oral intake, CHOL is absorbed in the intestine and packaged into chylomicrons which are transported to the liver, further processed, and distributed throughout the body[23].
Approximately 70 % of total body cholesterol is derived from *de novo* synthesis, whereas about 30 % is obtained from the diet, however this proportion differs among individuals due to genetics and dietary habits[23]. The European Food Safety agency (EFSA) reported an average daily CHOL intake of 200-550 mg per day for adults in Europe, or 2.86 – 7.86 mg/kg bw per day for 70 kg[24]. The estimated concentration in the small intestine is in the range of 1.90 g/L and 5.24 g/L. The values are based on the average small intestinal volume of 105 mL under fasting conditions and the average daily CHOL intake[22].

2.3 Phytoestrogens

Phytoestrogens are naturally occurring plant compounds that can be found in corn, oats, wheat, soybeans and barley. They can interact with the nuclear hormone receptors ERα and ERβ due to their structural similarities with estrogen[41]. Phytoestrogens may exhibit either agonistic or antagonistic effects on these receptors, which is determined by their bioavailability and concentration[41]. Their estrogenic activity might lead to endocrine disrupting properties and possible adverse health effects, which should be considered since phytoestrogens are used for their beneficial properties in pharmaceutical applications. In menopause, phytoestrogens are applied as therapeutics against symptoms such as hot flushes, and furthermore as supplements against breast cancer, prostate cancer, osteoporosis, type 2 diabetes, cardiovascular disease and obesity[42].

Phytoestrogens can be divided into the classes isoflavonoids, flavones, flavanols, flavanones, stilbenes, lignans and coumestans[41]. This study focuses on three phytoestrogens: genistein, daidzein and chrysin. Genistein and daidzein are classified as isoflavonoids, whereas chrysin belongs to the flavone class[41].

2.3.1 Genistein

Genistein (Genisteol 4',5,7-Trihydroxyisoflavone)[26] is part of the isoflavone class and is naturally present in legumes, fruits, seeds, and vegetables, for instance in broccoli, cauliflower, sunflower, clover seeds and sprouts[25]. The legume species soy presents the major source of genistein (GEN) through soybeans and some soy products (1.9-18.5 µg/g) and fermented soy such as in miso (38.5-230 µg/g)[25]. The anti-oxidative, anti-carcinogenic and anti-proliferative properties of GEN are reason for their application as food supplements, leading to strongly increased oral intakes compared to dietary levels[43]. After oral intake, GEN is rapidly absorbed in the intestine via passive diffusion, with high inter-individual variations. The glycosidic form of GEN, genistein, is hydrolyzed through phase 1 metabolism first to be absorbed. The majority of GEN is transformed into glucuronides and sulphates via phase 2 metabolism in intestine and liver. The conjugation products enter enterohepatic circulation and may be further metabolized, resulting in a minority of GEN present in its free form in the plasma[26,44]. According to a dietary study of Nordic Council of Ministers in 2020, the estimated average exposure to GEN is 0.2 mg/kg bw per day[26]. This corresponds to 14 mg/day for a 70 kg adult and 133.3 mg/L in the small intestine under fasting conditions (105 mL)[22].

2.3.2 Daidzein

Daidzein (Daidzeol 7,4'-Dihydroxyisoflavone)[26] belongs to the class of isoflavones and is naturally present in soybeans, alfalfa, red lentils, kudzu, red clover and other legumes, mostly in its glycosidic form. As for GEN, the primary source for daidzein (DAI) is in soybeans, with an average concentration of 0.1 – 5 mg/g[27]. After oral intake, DAI is absorbed in the intestine, and conjugated in the liver to glucuronides and sulfates, which are transported back over the bile into the intestine. Colon bacteria further metabolize DAI conjugates to equol. The extent of equol production in the colon shows high inter-individual variations, due to differences in the composition of gut microbiota[27]. Another more common pathway is the hydrolysis of DAI to O-desmethylan golensin (ODMA) by bacterial enzymes[27]. The glycosidic form of DAI, daidzin, must first be hydrolyzed by bacteria or enzymes in the intestine before being absorbed and undergoing the described pathway[45].

The estimated average exposure to DAI is 0.2 mg/kg bw per day, according to a dietary study by the Nordic Council of Ministers in 2020[26]. For a 70 kg adult, this equates 14 mg/day and 133.3 mg/L in the small intestine under fasting conditions (105 mL average volume)[22].

2.3.3 Chrysin

As part of the flavone class, chrysin (5,7-dihydroxyflavone) is another naturally occurring compound in the plant world. Chrysin (CHRY) is found in propolis, as well as in honey, particularly in forest honey (5.3 mg/kg)[28]. The blue passionflower also contains CHRY. Like other phytoestrogens, the compound has beneficial health effects like anticancer, anti-inflammatory and antioxidant properties[28]. Its therapeutic potential is limited by low oral bioavailability. CHRY is barely absorbed but effectively conjugated with glucuronides and sulfates[28].

The estimated average CHRY intake in the european diet is 70.6 µg/day, or 1 µg/kg bw per day for 70 kg adults. This value was calculated taking into account the concentration of CHRY in forest honey (5.3 mg/kg)[28], as its primary source, as well as the average consumption of forest honey (13.32 g/day), according to the EFSA Food Consumption Data Base from 2022[29]. The estimated concentration in the small intestine under fasting conditions is 0.672 mg/L (based on the average intake, 105 mL small intestinal volume)[22].

2.4 Mycoestrogens

Not only plants naturally contain compounds with endocrine active properties, but also the class of filamentous fungi produces secondary metabolites with endocrine activity, known as mycoestrogens. Contamination with these mycotoxins can be present in oats, rice, wheat, maize, hay and other crops[2]. Environmental factors such as elevated CO_2 levels and high humidity contribute to fungal formation on crops, making fungi contaminations a topic with growing relevance in respect of climate change[46]. While *Alternaria* fungi are responsible for the occurrence of alternariol, the mycoestrogen zearalenone derives from *Fusarium* species[2].

2.4.1 Zearalenone

Zearalenone (ZEN) is found in cereals and its processed products but can also be present in animal derived products if contaminated feed is consumed. Due to accumulation in the food chain, contaminations with ZEN may be present not only in maize, rice, soybeans, rye, sesame seed, barley, wheat, beer and flour but also in eggs, milk and meat[2]. After oral intake, ZEN is rapidly absorbed in the intestine and enzymatically reduced to α-zearalenol and β-zearalenol via phase I metabolism. Conjugation of ZEN and its metabolites with glucuronic acids and sulphates via phase II metabolism

takes place in intestine, liver and likely also in other tissues[30]. In addition, ZEN quinones may be formed via catecholes and tend to undergo redox cycling and can affect biomolecules[30]. Next to various toxic effects, ZEN is known for its endocrine activity. Due to the structural und functional similarity to E2, ZEN shows affinity to ER, with higher preference for ERα[47]. According to studies conducted across 19 European countries from 2005 to 2010, the estimated chronic total oral exposure with ZEN lies between 2.4 and 29 ng/kg bw per day (minimum LB - maximum UB) for adults. Toddlers between 12 and 36 months experience the highest chronic exposure of all age groups with 9.3 - 100 ng/kg bw per day. In 2011, the EFSA Panel for Contaminants in the Food Chain (CONTAM Panel) defined a TDI for ZEN of 0.25 µg/kg bw per day, or 17.5 µg/day for 70 kg adults[30]. The estimated concentration of zearalenone in the small intestine, calculated with the TDI and the average small intestinal volume of 105 mL under fasting conditions, is 166.7 µg/L[22].

2.5 Deoxynivalenol

Deoxynivalenol (DON) is part of the class of mycotoxins and is also produced by *Fusarium* fungi, yet without showing endocrine activity[48]. Instead, DON is known for its ability to impair eukaryotic protein synthesis via interference with ribosomes at the peptidyl transferase site[49,50]. DON is present in cereals like maize, oat grains, wheat, rice, rye, barley and derived food products such as bread and rolls. Animal feed is frequently contaminated with DON at even higher levels. In animals, DON leads to acute gastrointestinal symptoms and shows chronic effects such as anorexia, reduced nutritional efficacy and inhibition of weight gain. Similar symptoms are observed in humans[31]. According to *in vitro* and *in vivo* studies in humans, ingested DON is mostly conjugated to glucuronides and, to lesser extent, de-epoxidized to DOM-1[51]. The Scientific Committee for Food (SCF) established a TDI of 1 µg/kg bw per day for DON in 2001, which is accurate until now. In fungi, DON is accompanied by its acetyl derivates such as 3-acetyl-DON and 15-acetyl DON, which were incorporated in the TDI in 2010 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)[31]. Based on the TDI, the concentration in the small intestine is expected to be 2.25 µM after oral DON intake under fasting conditions and an intestinal volume of 105 mL[22]. According to European dietary exposure assessments by EFSA, certain population groups had reached or exceeded the set TDI for DON. The highest average chronic dietary exposure was observed for infants, toddlers and other children with 0.54 – 1.02 µg/kg bw per day (upper bound). Adolescents, adults and elder population groups were exposed to DON at an average of 0.22 – 0.58 µg/kg bw per day (upper bound)[31].

2.6 Tight Junction Protein CLDN4

To ensure an effective barrier and gate function, epithelial cells are connected via tight junction proteins (TJs) which divide apical and basolateral membranes and are important regulators of paracellular transport of ions and dissolved substances[52]. In addition, TJs contribute to signal recognition, transduction and response in cells. For intercellular adhesion, the basolateral membrane contains also other protein complexes, such as adherens junctions (AJs) and desmosomes[53]. Gap junctions serve for the communication between cells via passive diffusion through channels[54].

TJs are located near the apical side of the cells with more than 40 different proteins known to date. TJs are made of integral proteins which are connected to cytoplasmic plaque proteins, such as zonula occludens and cingulin, that are further linked to the actin cytoskeleton. Next to occludins, tricellulins and junctional adhesion molecules (JAMs), claudins serve as important integral proteins. The schematic representation of the structure and localization of TJs is shown in **Fig 2.1**[53]. Claudins are present in humans with more than 26 different types. They consists of two extracellular loops (EC1 and EC2), four transmembrane domains, an amino tail and a carboxyl tail with PDZ-motif for connection to other TJ proteins[55,56]. Both extracellular loops enable an intercellular *cis-, trans-* interaction, the paracellular barrier and pores are formed by the extracellular loop EC1 only[56]. The distribution of claudin types varies between tissues and also molecules such as hormones can influence claudin location and expression[55]. Since claudins are crucial for barrier integrity and polarity of epithelial cells, undesired changes in the expression or localization of the protein may lead to adverse effects on homeostasis. In tumors, up- and downregulation of claudin expression was observed, depending on the tissue. Keeping in mind the important roles of claudin in the cell, alterations in the expression of the integral proteins might also influence tumor progression[57].

Breast cancer[58], ovarian cancer[59], gastric cancer[60], colorectal cancer [61] and various other cancer tissues showed an increased expression of claudin 4 (CLDN4). The protein CLDN4 is one of the most present TJ proteins in epithelial cells including the intestines and lungs. Upregulation of CLDN4 in cancer can be triggered by various factors, such as epigenetics, inflammation processes and growth factors. For instance, reactive oxygen species leading to oxidative stress in the body seem to increase CLDN4 expression via NF-κB inactivation[62].

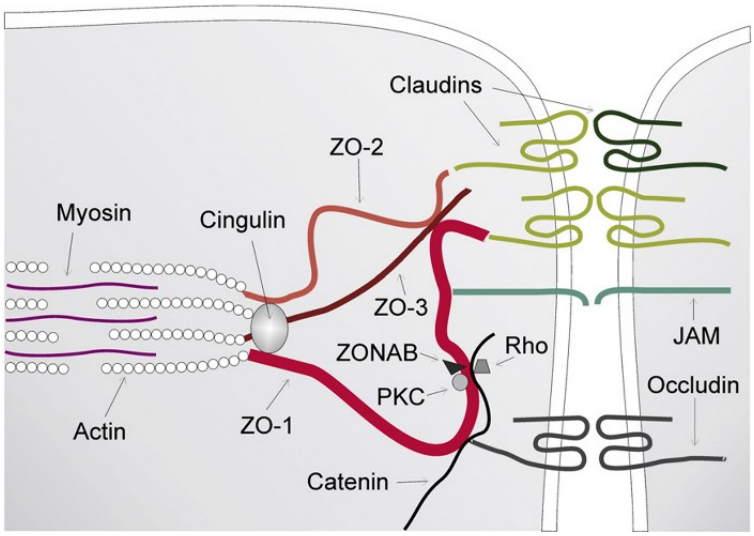


Fig 2.1 Schematic structure of TJ proteins: Claudin, zonula occludens (ZO-1, ZO-2, ZO-3), occludin, junctional adhesion molecules (JAM), cingulin, zonula occuldens-1 associated nucleic acid binding protein (ZONAB), protein kinase C (PKC), small GTPases (Rho) (Taken from: Kosińska and Andlauer 2013[53])

3 Materials & Methods

3.1 Materials

Applied materials are shown in the Supplementary Table.

3.2 Methods

3.2.1 PBPK-model

In this study, physiologically based pharmacokinetic modelling (PBPK) with the Integrated Chemical Environment (ICE)[63] tool was applied to obtain a first reference estimate of the tissue-specific concentration for the test substances after oral exposure. The predictions rely on experimentally measured parameters and in silico estimations. Absorption, distribution, metabolism, and excretion of the substances are therefore considered. The daily doses for each substance used as inputs were derived from literature, as described in the theoretical section, and are summarized also in **Tab 3.1**. Therefore ADI, TDI and average daily intake were considered, also in dependency of the substance type

and current available data. The average daily intake was applied for calculation of the concentrations for CHOL, GEN, DAI, CHRY, ZEN and DON. For E2 the ADI was used, due to scarce data availability for the average daily intake. For the purpose of the simulations, oral doses were given every 24 hours for seven consecutive days (Solve_pbtk).

Chemical Name	CAS Number	Oral daily dose
17β-Estradiol (E2)	50-28-2	50 ng/kg bw
Cholesterol (CHOL)	57-88-5	7.86 mg/kg bw
Genistein (GEN)	446-72-0	0.2 mg/kg bw
Daidzein (DAI)	486-66-8	0.2 mg/kg bw
Chrysin (CHRY)	480-40-0	1 µg/kg bw
Zearalenone (ZEN)	17924-92-4	29 ng/kg bw
Deoxynivalenol (DON)	51481-10-8	0.58 µg/kg bw

Tab 3.1 Summary: chemical names, CAS numbers, oral daily doses applied in PBPK-model.

3.2.1.1 Cell Cultivation

Caco-2, HT29 and HT29-MTX-E12 cells were cultured in DMEM (high glucose, 4.5 g/L, Gibco, REF 21063–029) supplemented with fetal bovine serum (10%), penicillin / streptomycin (1%), non-essential amino acids (1 %) and L-glutamine (1 %). All cell lines were held in humidified incubators at 37 °C and 5 % CO_2. Caco-2 cells were cultured in T75 flasks, HT29 and HT29-MTX-E12 were maintained in T25 flasks.

Depending on confluency level, passaging was carried out every 2 to 4 days. After medium aspiration, cells were washed once with 5 mL PBS. For detachment of HT29 and HT29-MTX-E12 cells, 1 mL Trypsin-EDTA solution was added. For Caco-2 cells, 2 mL of Trypsin-EDTA solution was used. After 5-10 min of incubation at 37 °C and 5 % CO_2 and upon confirmation of cell detachment under a light microscope, 5 mL of medium was added to the flask, homogenized and transferred to a 10 mL tube. Cell concentration was determined according to the counting procedure described in 3.2.1.2. For passaging, 700 000 cells of HT29 or HT29-MTX were transferred back in T25 flasks and 1400 000 cells of Caco-2 were added in T75 flasks. After addition of 10-15 mL medium, the flasks were returned to the incubator.

3.2.1.2 Cell Counting

For cell counting, trypan blue–PBS solution (1:10) was used as a contrast agent, staining dead cells blue while living cells remain white. Cell suspensions were diluted with trypan blue-PBS solution in the ratio

1:2 to 1:10 dependent on the confluency and the cell line. 10 µL of the diluted suspension was loaded into a Neubauer counting chamber covered with a cover slip. Under a light microscope, all cells within four large squares were counted. Cells on the upper and left outer lines were included, whereas cells on the lower and right outer lines were not counted. The cell concentration was calculated by multiplying the average amount of cells per large square by the dilution factor and by multiplication factor as shown in **Eq 3.1**. The multiplication factor of 10^4 is based on the relation of the volume of the large square to 1 mL.

$$\textbf{concentration}\ [\textbf{cells/mL}] = \textbf{average cell count per large square} \times \textbf{dilution factor} \times \mathbf{10^4}$$

Eq 3.1

3.2.2 Treatment Preparation

Stock solutions were prepared by dissolving the substances GEN, DAI, CHRY, ZEN, DON in Dimethyl sulfoxide (DMSO), E2 was dissolved in Ethanol (EtOH) and Cholesterol was dissolved in ddH_2O. The stock solutions were further diluted with DMEM or NES (normal external solution)[64], dependent on the assay. The test solutions contained a maximum final concentration of 0.1 % DMSO, which was also applied to the solvent control.

3.2.3 Cytotoxicity

For evaluating the possible cytotoxic effects of the test substances, a CellTiter-Blue® assay was performed. The assay is based on the metabolic activity of viable cells and their ability to reduce CellTiter-Blue® reagent resazurin to resorufin, as shown in **Fig 3.1**[65]. Resazurin appears dark blue with little fluorescence, whereas the reduced form resorufin is pink and strongly fluorescent (560_{Ex} /590_{Em}). Due to the reduced capacity of non-viable cells to metabolize the CellTiter-Blue® agent resazurin, the fluorescent signal of resorufin correlates with the amount of viable cells[65].

Cells were seeded in 96-well plates at a density of 36 000 cells/well for HT29 and 27 000 cells/well for Caco-2. 24 hours of growth time were followed by medium aspiration and the addition of the test substances dissolved in culture medium (200 µL per well). The cells were incubated with the substances for 24 h. CellTiter-Blue® solution was diluted 1:10 with phenol red-free DMEM. After medium aspiration, 100 µL of CellTiter-Blue® were added to each well under dark conditions. After 1 h incubation time at 37 °C and 5 % CO_2 protected from light, 80 µL of the supernatant was transferred to a black, flat-

bottom 96-well plate. Fluorescence signal was measured at (560 nm_{Ex} / 590 nm_{Em}) using a plate reader. Each condition was tested on 3 biological replicates and technical triplicates. The fluorescence signal was normalized to solvent controls T/C (%).

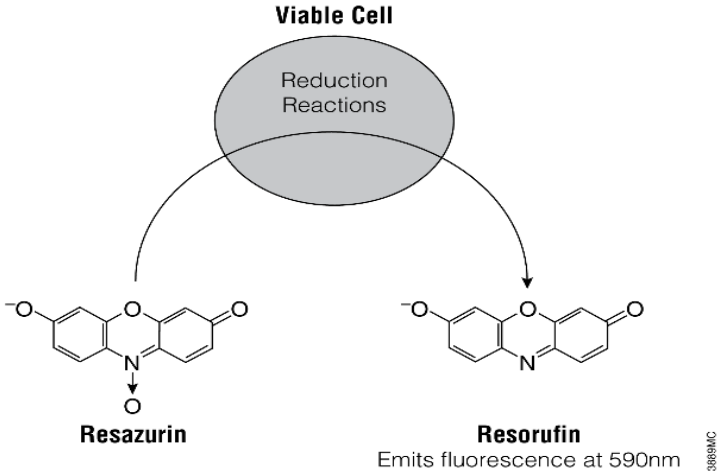


Fig 3.1 Reduction of resazurin to resorufin in viable cells. (Taken from: Promega: CellTiter-Blue® Cell Viability Assay 2023[65])

3.2.4 Membrane Fluidity

The membrane fluidity of Caco-2 cells under treatment conditions was tested using 1-pyrenedecanoic acid (PDA) following the procedure previously described by Del Favero et al., 2018[66]. The assay is based on the fluorescence properties of PDA. After excitation at 344 nm, the monomeric form of PDA emits at 375 nm (I_M), while the excimer form of PDA emits at 470 nm (I_E). After applying PDA on cells, the fluidity of the membrane dictates whether PDA remains in monomeric state or forms excimers.

Caco-2 (27 000 cells/well) were seeded in clear bottom 96-well black plates and grown for 24 h in the incubator. For the 10-min assay, the medium was replaced by PDA-NES solution (10 µM, 100 µL per well) under dark conditions and incubated at 37 °C for 1 h. Treatments were dissolved in NES and loaded on the cells after medium aspiration. After 10 min incubation at 37 °C, fluorescence intensity was measured (Ex: 344, 344 nm, Em: 375, 470 nm). For the 24-h assay, treatments were dissolved in culture medium and applied for 24 h hours. After the incubation time, PDA-NES solution was added (10 µM, 100 µL per well) under dark conditions and incubated for 1 h. PDA-NES was replaced by NES and kept at 37 °C for 10 min and fluorescence intensity was measured (Ex: 344, 344 nm, Em: 375, 470 nm).

Membrane fluidity was calculated by the ratio of (I_E / I_M). Results were shown as percentage Test over Control (T/C * 100) with n=3 biological replicates in technical triplicates.

3.2.5 Tight Junction Expression

3.2.5.1 Seeding of Co-culture

For the co-culture, Caco-2 and HT29-MTX-E12 were seeded at a 9:1 ratio in 8 well slides, resulting in a cell density of 85 000 cells/cm^2. 7 days-differentiation period yielded a cell monolayer with mucus, which mimics the environment in the intestinal epithelium. Culture medium was replaced every 2-3 days throughout the differentiation period.

3.2.5.2 Mucus Reduction and Treatment

During the differentiation time of the co-culture Caco-2/HT29-MTX-E12 a mucus layer was produced. For additional observations, the mucus layer was removed using N-acetyl-cysteine, as previously described by Behrens et al., 2002[67] with slight modifications. Medium was replaced by N-acetyl-L-cysteine (10 mM, 200 µL per well) and shaken for 30 min at 220 rpm in the incubator. Thereafter, N-acetyl-L-cysteine was renewed, and the shaking was repeated. The cells were washed three times with culture medium before the incubation with test substances. The differentiated co-culture with or without applied mucus reduction was incubated with the substances E2, CHOL, GEN, DAI, CHRY, ZEN and DON for 24 h (200 µL per well).

3.2.5.3 Immunofluorescence Staining

After incubation with the test substances, cells were fixed with MeOH/AcOH (10:1) overnight at 4°C (150 µL per well). Cells were washed twice with PBS-A, permeabilized with 0.2 % Triton X-100 in PBS-A for 15 min at RT (250 µL/well). Thereafter, unspecific binding sites were blocked with 2 % donkey serum in PBS-A for 1 h at RT (250 µL/well). CLDN4 monoclonal antibody (1:500 in PBS-A) was incubated overnight at 4°C (200 µL/well). Cells were washed 3 times with 0.05 % Triton X- 100 (in PBS-A) and twice with PBS-A, on a rocking table for 10 min and 5 min, respectively. Post fixation with MeOH/AcOH (10:1, 200 µL/well) for 15 min at RT was followed by washing with PBS-A. After washing twice with PBS-A, 2-3 drops of DAPI containing mounting media were added per well. The sealed slides were stored at 4°C.

3.2.5.4 Quantification

Confocal images of the CLDN4 stained co-culture were taken with LSM 710 (Zeiss) at 63 x magnification. For each of the 3 biological replicates, three 2D-images and one z-stack were acquired. The evaluation was done using FIJI and data were compared and displayed with the help of the Origin Pro software. A schematic representation of the cell monolayer (light red), the nuclei (blue) and the CLDN4 tight junctions (red) is shown in **Fig 3.2**. CLDN4 thickness was evaluated by measuring the signal thickness as shown in (1, **Fig 3.2**) 15 randomly chosen tight junctions were evaluated per 2D-image, with 3 images per biological triplicates, resulting in 135 distance measurements per condition. The CDLN4 signal height was quantified from the height of the CLDN4 signal in the z-stacks as shown in (2, **Fig 3.2**.) from four quarters per z-stack with n=3 z-stacks from n=3 biological replicates. The z-distance is defined as the distance between the plane of maximum tight junction expression and the midpoint of the nuclei as shown in (3, **Fig 3.2**). Four quarters were measured per z-stack with n=3 z-stacks from n=3 biological replicates. The morphology of the nuclei (4, **Fig 3.2**.) was analyzed via assessing relative area, circularity, aspect ratio, roundness and solidity. 15 nuclei per biological replicate were randomly chosen, resulting in n=45 nuclei per condition.

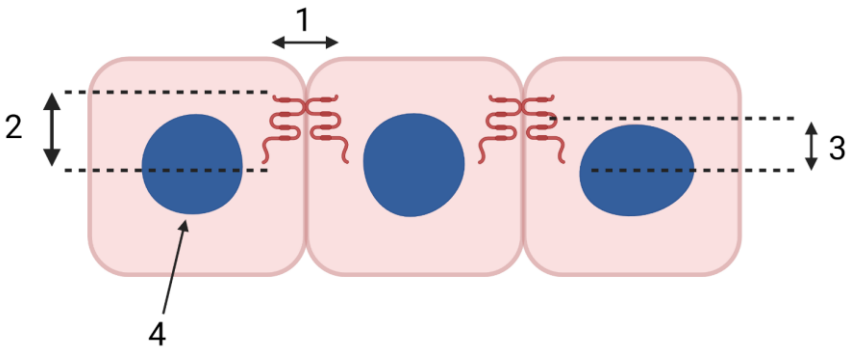


Fig 3.2 Schematic representation of tight junction evaluation,1) Thickness, 2) CDLN4 signal height, 3) Z-distance, 4) Nuclei analysis. (Created with Biorender.com.)

4 Results

4.1 PBPK-model

For first estimating the concentration of the test substances in the gut after oral intake, an open source platform for physiologically based pharmacokinetic modelling (PBPK)[63] was applied. The model, concentrations and parameters used are described in section 3.2.1. After assuming daily oral intake for seven consecutive days, a concentration profile for each substance and for each tissue was predicted by the software. The maximum substance concentration Cmax [µM] for each tissue throughout the simulation was plotted with the Origin software. For the endogenous substance 17β-estradiol, 50 ng/kg bw were applied as daily oral dose, which resulted in the concentration profile shown in **Fig 4.1.** The highest C_{max} for E2 was predicted in plasma, the lowest C_{max} in the lung, followed by the gut with C_{max} of 0.67 nM. The initial oral concentration applied for CHOL was 7.86 mg/kg bw per day. C_{max} for CHOL was highest in the gut tissue (400.7 µM) and lowest in the plasma, as shown in panel B**.** The simulation for GEN (0.2 mg/kg bw per day) resulted in the C_{max} of 2.83 µM for the gut tissue, only topped by the concentration in liver tissue (panel C). According to the applied model, daily oral exposure with DAI (0.2 mg/kg bw per day), leads to the highest C_{max} in the liver followed by C_{max} in the gut (5.96 µM) as shown in panel D. The tissue specific distribution after oral exposure with CHRY (1 µg/kg bw per day) is displayed in panel E**.** High concentrations were predicted in the liver and plasma, moderate concentrations (4.17 nM) appeared in the gut. All Phytoestrogens GEN, DAI, CHRY showed the lowest C_{max} in the lung tissue. Oral exposure with mycotoxin ZEN (29 ng/kg bw per day, panel F) led to highest concentration levels in the gut with 0.89 nM, followed by the liver, while low concentrations were present in all other tissues.

For direct comparison, the substance concentrations present in the gut were plotted in **Fig 4.2**. The seven-day simulation with oral exposure every 24 hours resulted in a time dependent concentration profile for each compound. Therefore, modelled distribution, accumulation, metabolism and excretion could be further observed specifically in the tissue most relevant for the research question. The endogenous substances E2 and CHOL showed strong and moderate accumulation in the gut tissue, respectively. According to the prediction, the phytoestrogens GEN, DAI and CHRY as well as the mycotoxin ZEN showed little to no accumulation and were mostly eliminated within 24 h.

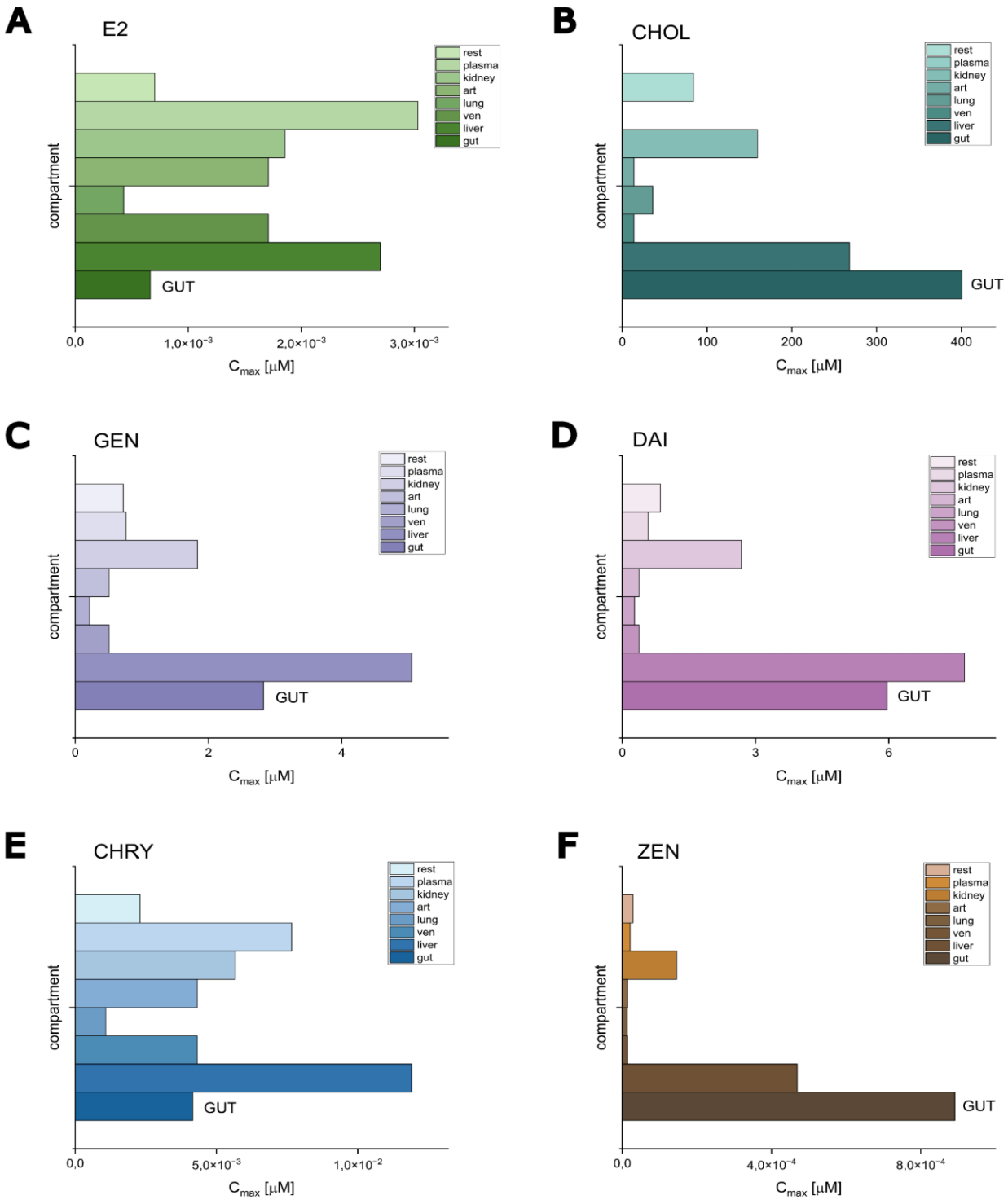


Fig 4.1 Tissue specific concentration profiles C_{max} [µM] via PBPK-modelling via ICE (A) C_{max} after E2 exposure of 50 ng/kg bw per day (B) C_{max} after CHOL exposure of 7.86 mg/kg bw per day, (C) C_{max} after GEN exposure of 0.2 mg/kg bw per day, (D) C_{max} after DAI exposure of 0.2 mg/kg bw per day , (E) C_{max} after CHRY exposure of 1 µg/kg bw per day (F) C_{max} after ZEN exposure of 29 ng/kg bw per day.

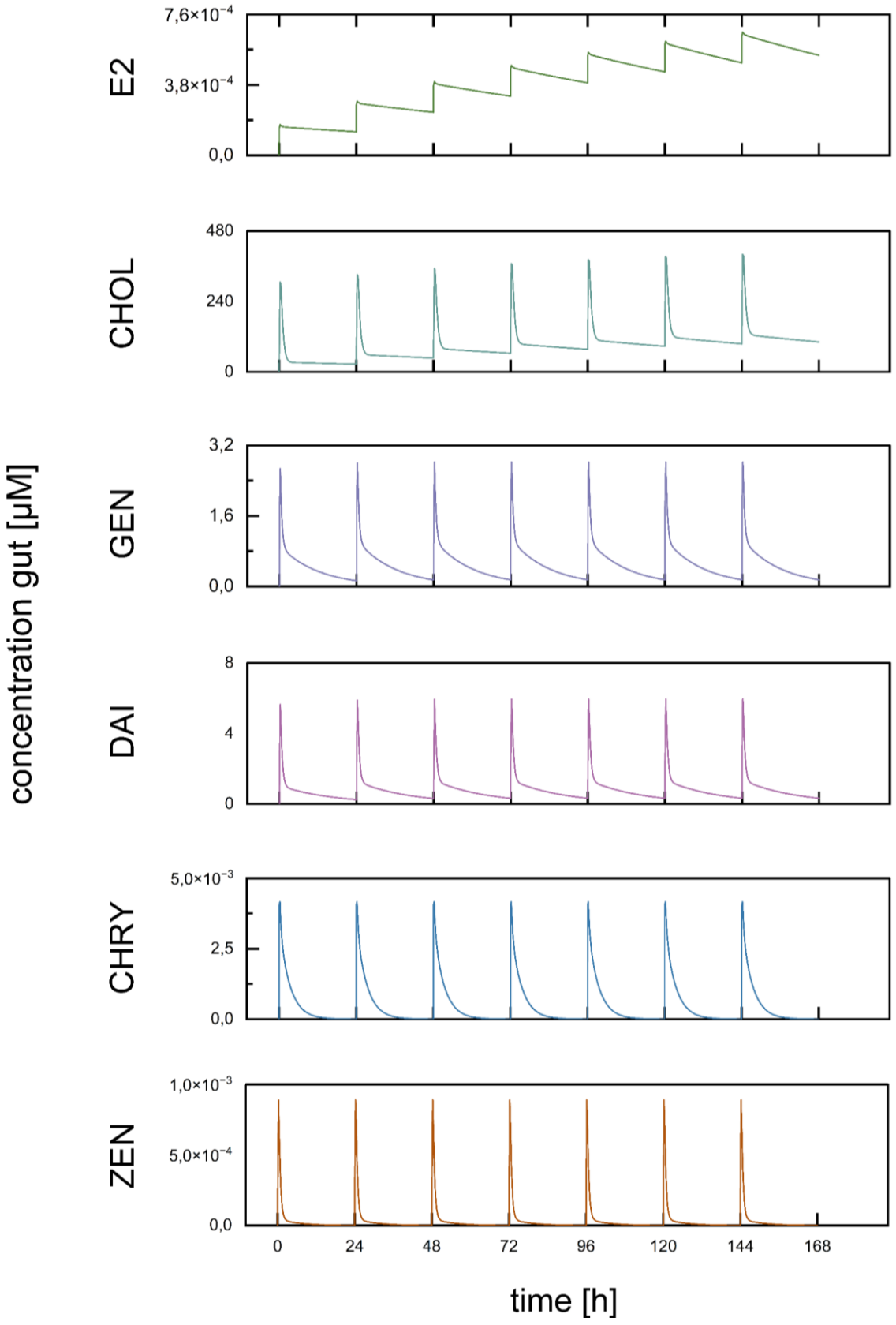


Fig 4.2 PBPK-model via ICE: time dependent concentration profile [µM] in gut tissue after daily oral exposure with E2 (50 ng/kg bw per day), CHOL (7.86 mg/kg bw per day), GEN (0.2 mg/kg bw per day), DAI (0.2 mg/kg bw per day), CHRY (1 µg/kg bw per day), ZEN (29 ng/kg bw), simulation length: 7 days.

4.2 Cytotoxicity

Possible cytotoxic effects of the test substances on both Caco-2 and HT29 cell lines were investigated via CellTiter-Blue® Assay. The cell viability [%] after 24 h exposure with E2 (0.1 - 10 nM), CHOL (0.01 - 1 µg/mL), GEN (0.1 – 10 µM), DAI (0.1 – 10 µM), CHRY (0.1 – 10 µM) and ZEN (1 - 100 nM) is shown in **Fig 4.3**. Incubation with the substances resulted in no significant changes in cell viability in Caco-2 cells (panel A) and in HT29 cells (panel B).

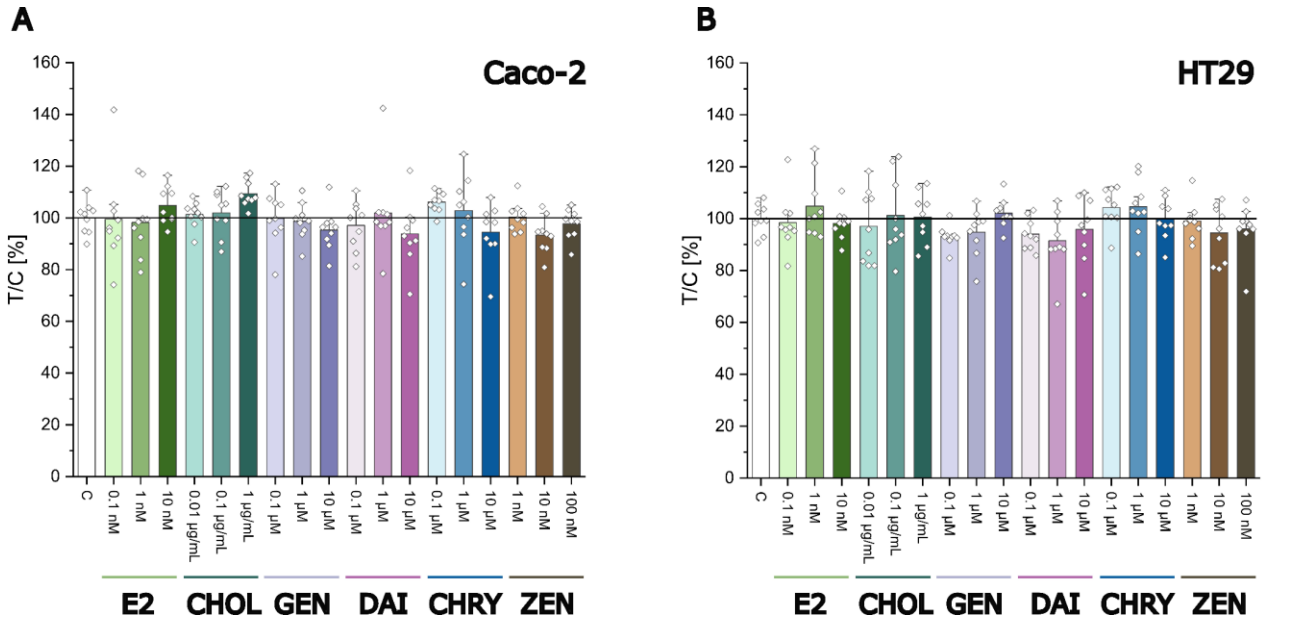


Fig 4.3 Cell viability after treatment with EACs in Caco-2 and HT29 cells. Cytotoxic effects of endocrine active compounds on Caco-2 (A) and HT29 (B) via CellTiter-Blue® Assay. Cell viability [%] after 24 h exposure with E2 (0.1 - 10 nM), CHOL (0.01 - 1 µg/mL), GEN (0.1 – 10 µM), DAI (0.1 – 10 µM), CHRY (0.1 – 10 µM) and ZEN (1 - 100 nM) compared to control. Data points display the individual technical replicates, mean values +- SD summarized the outcome of at least 3 independent biological replicates, performed in technical triplicates.

4.3 Membrane Fluidity

Since the fluidity of cell membranes can affect the function of membrane proteins[18], the influence of E2, CHOL, GEN, DAI, CHRY and ZEN on membrane fluidity is of significant relevance in this study as it might affect the biophysical environment surrounding the TJ proteins. Changes in membrane fluidity of Caco-2 cells after 10 min and 24 h incubation with E2 (1 nM), CHOL (0.1 µg/mL), GEN (1 µM), DAI (1 µM), CHRY (1 µM) and ZEN (10 nM) were assessed via a PDA-based assay as described in section 3.2.4. H_2O_2 (1 mM) and palmitic acid (250 µM)[68] were included in the experimental layout as positive controls for short and long term incubations. Incubation with H_2O_2 for 10 min led to a significant decrease in membrane fluidity, validating the assay shown in **Fig 4.4** panel A. 10 min treatment with E2 and GEN significantly ($p < 0.01$) decreased membrane fluidity compared to controls. Membrane fluidity of Caco-2 cells was also decreased after 10 min exposure with ZEN ($p < 0.05$) whereas CHOL exposure increased membrane fluidity. DAI and CHRY did not induce relevant changes in membrane fluidity.

Palmitic acid significantly ($p < 0.001$) decreased membrane fluidity in Caco-2 cells after 24 h of exposure, as shown in Panel B, **Fig 4.4**. In contrast to the 10 min assay, E2 exposure increased membrane fluidity in the 24 h experiment. CHRY also led to a significant increase in membrane fluidity ($p < 0.01$). CHOL, GEN, DAI and ZEN did not modify membrane fluidity at 24h of incubation.

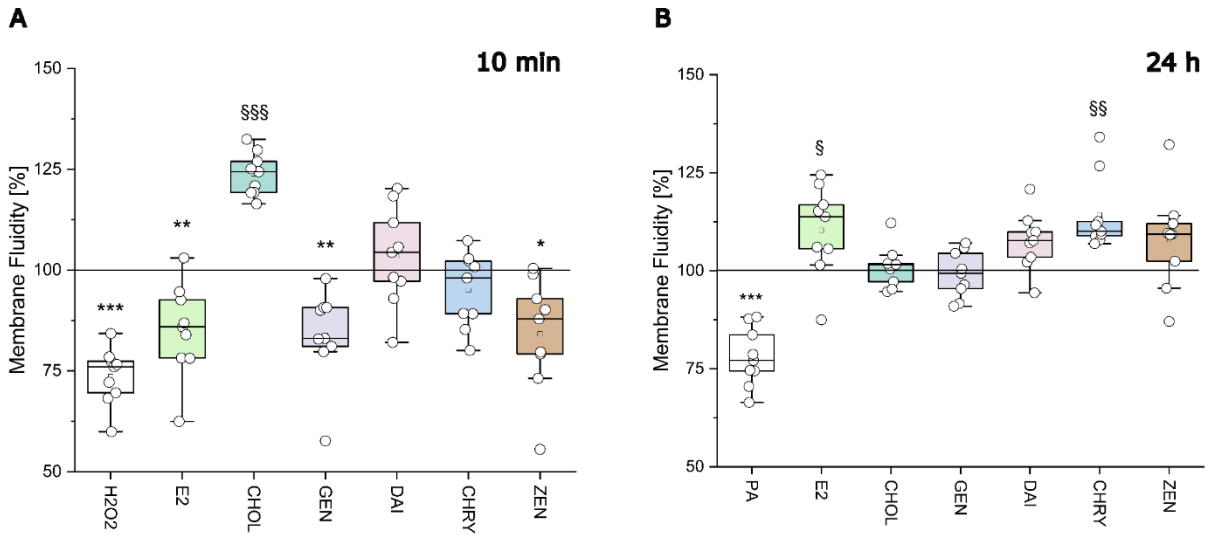


Fig 4.4 Endocrine active substances induce change in membrane fluidity in Caco-2 cells. Membrane fluidity [%] via PDA-assay after 10 min (A) and 24 h (B) exposure to E2 (1 nM), CHOL (0.1 µg/mL), GEN (1 µM), DAI (1 µM), CHRY (1 µM), ZEN (10 nM), with */§ $p < 0.05$, **/§§ $p < 0.01$, ***/§§§ $p < 0.001$ compared to control, Student's t-test (Welch correction).

4.4 Immunofluorescence Staining

4.4.1 Nuclei Analysis

To investigate possible effects of the test compounds on the arrangement of the Caco-2/HT29-MTX-E12 cell monolayer, the morphology of the nuclei was investigated. After 24 h exposure with either E2 (1 nM), CHOL (0.1 µg/mL), GEN (1 µM), DAI (1 µM), CHRY (1 µM) or ZEN (10 nM) the cells were assessed on the morphology of the nuclei by evaluation of relative area, solidity, roundness, circularity and aspect ratio. The morphological markers are plotted in **Fig 4.5** for cells with mucus and cells after mucus reduction. 24 h Incubation with GEN and ZEN led to a significant increase in relative area for the co-culture with mucus, as shown in **Fig 4.5** panel A and panel C, with $p < 0.001$ and $p < 0.01$, respectively. Exposure to CHOL induced a slight increase in aspect ratio and a decrease in nuclei solidity (both $p < 0.05$). For the cell layer after mucus removal (panel B, D) a decrease in the relative area of the nuclei was induced by ZEN exposure ($p < 0.05$, panel D). E2 significantly increased the circularity, the roundness and the solidity of the nuclei ($p < 0.01$, $p < 0.05$, $p < 0.05$). Exposure to CHRY led to a slight increase in nuclei circularity ($p < 0.05$) for the cell model with reduced mucus.

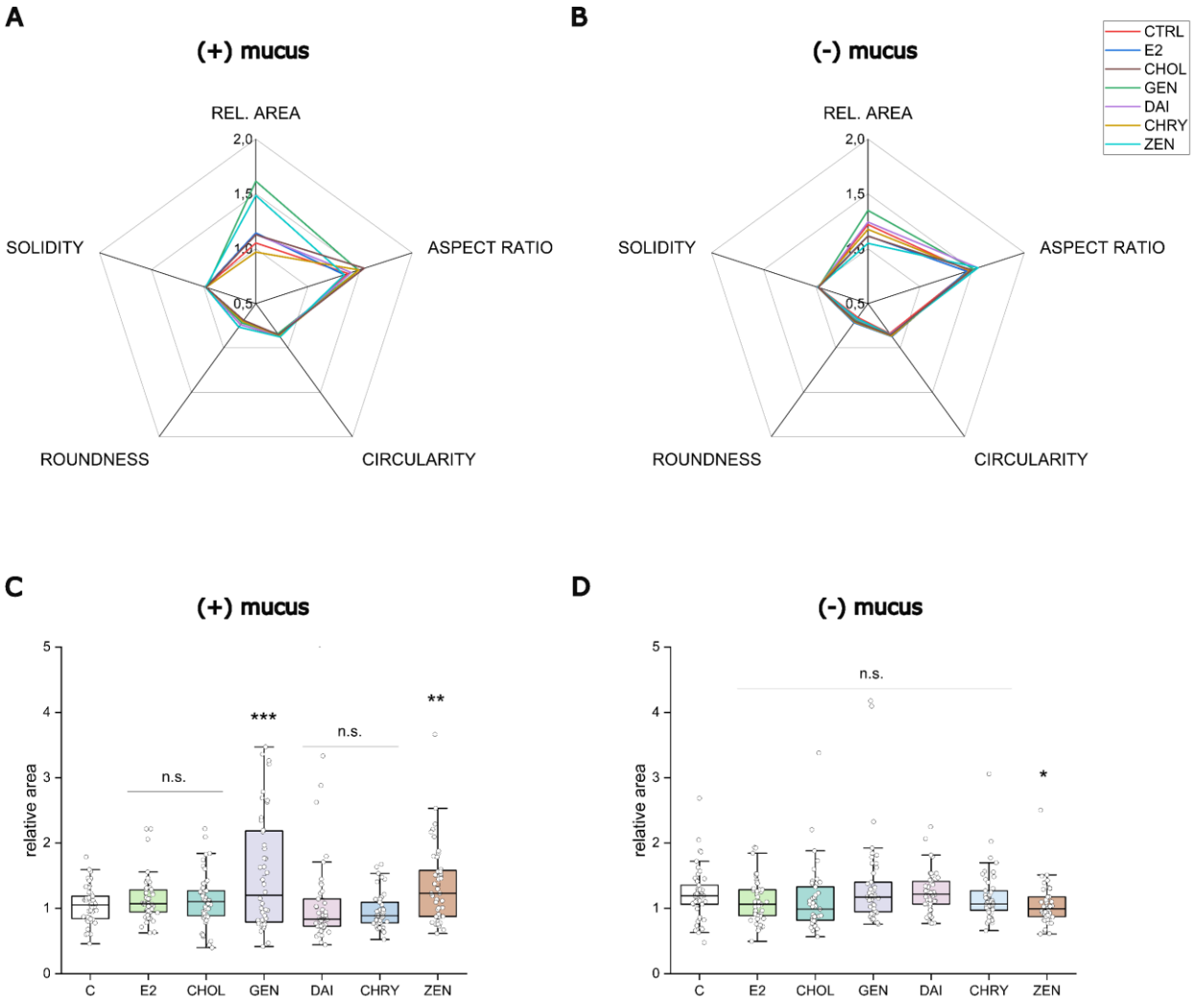


Fig 4.5 Nuclei morphology of Caco-2/HT29-MTX-E12 co-culture treated with endocrine active compounds. 24 h exposure to E2 (1 nM), CHOL (0.1 µg/mL), GEN (1 µM), DAI (1 µM), CHRY (1 µM), ZEN (10 nM) on morphological nuclear descriptors (A, B) relative area, aspect ratio, circularity, roundness, solidity of nuclei in co-culture with mucus (A) and reduced mucus (B) n=45 nuclei from n=3 biological replicates, (C, D) relative area of nuclei with mucus (C) and without mucus (D) n=45 nuclei from n=3 biological replicates with *p < 0.05, **p < 0.01, ***p < 0.001, Student's test (Welch correction).

4.4.2 CLDN4 Expression

CLDN4 is a tight junction protein present in duodenum, ileum and other parts of the gastrointestinal tract[54], and its expression contributes to barrier integrity[55,69,70]. Since altered formation and mislocalization of claudins are associated with various pathological conditions[71,72], the effect of the test substances on CLDN4 expression, distribution, and on the CLDN4 signal height were of particular interest in this study. The mentioned parameters were assessed following immunofluorescence staining of CLDN4. The effect of 24 h exposure with E2 (1 nM), CHOL (0.1 µg/mL), GEN (1 µM), DAI (1 µM), CHRY (1 µM) or ZEN (10 nM) on CLDN4 was investigated. **Fig 4.6** panel A shows representative 2 D images of CLDN4 in the co-culture with and without mucus after treatment with the compounds of interest. The thickness of CLDN4 tight junction is shown in **Fig 4.6** as test over control [%] for conditions with mucus (panel B) and after mucus reduction (panel C). In the co-culture with mucus, treatment with E2, CHOL and CHRY induced a significant ($p < 0.001$) decrease in CLDN4 thickness compared to control. GEN and DAI also resulted in a reduced CLDN4 thickness, whereas ZEN showed no significant effect.

The effect on CLDN4 thickness after treatment of the co-culture under mucus reduced conditions are shown in panel C (**Fig 4.6**). A significant decrease in signal thickness was observed for E2 and CHRY ($p < 0.001$) and for CHOL ($p < 0.01$) compared to control. GEN, DAI and ZEN induced no variations in comparison to controls.

A representative z-stack of the Caco-2/HT29-MTX-E12 coculture with CLDN4 in red and nuclei in blue is shown in **Fig 4.7** panel A. The CDLN4 signal height, representing the CDLN4 thickness of the monolayer in the z-axis, is shown in panel B and C. Compared to control, E2, CHOL and CHRY exposed cells showed a decreased CDLN4 signal height in the co-culture with mucus (panel B, **Fig 4.7**). After mucus reduction, only E2 induced a significant decrease in the height of the cell layer (panel C, **Fig 4.7**).

The distance between the CLDN4 tight junction and the nuclei along the z-axis, defined as the z-distance, is presented in **Fig 4.7** (panel D and E). The co-culture model with mucus showed an increase in the z-distance upon GEN and DAI exposure ($p < 0.05$). Under mucus reduced conditions, a significant increase in z-distance was observed only after CHRY treatment ($p < 0.05$).

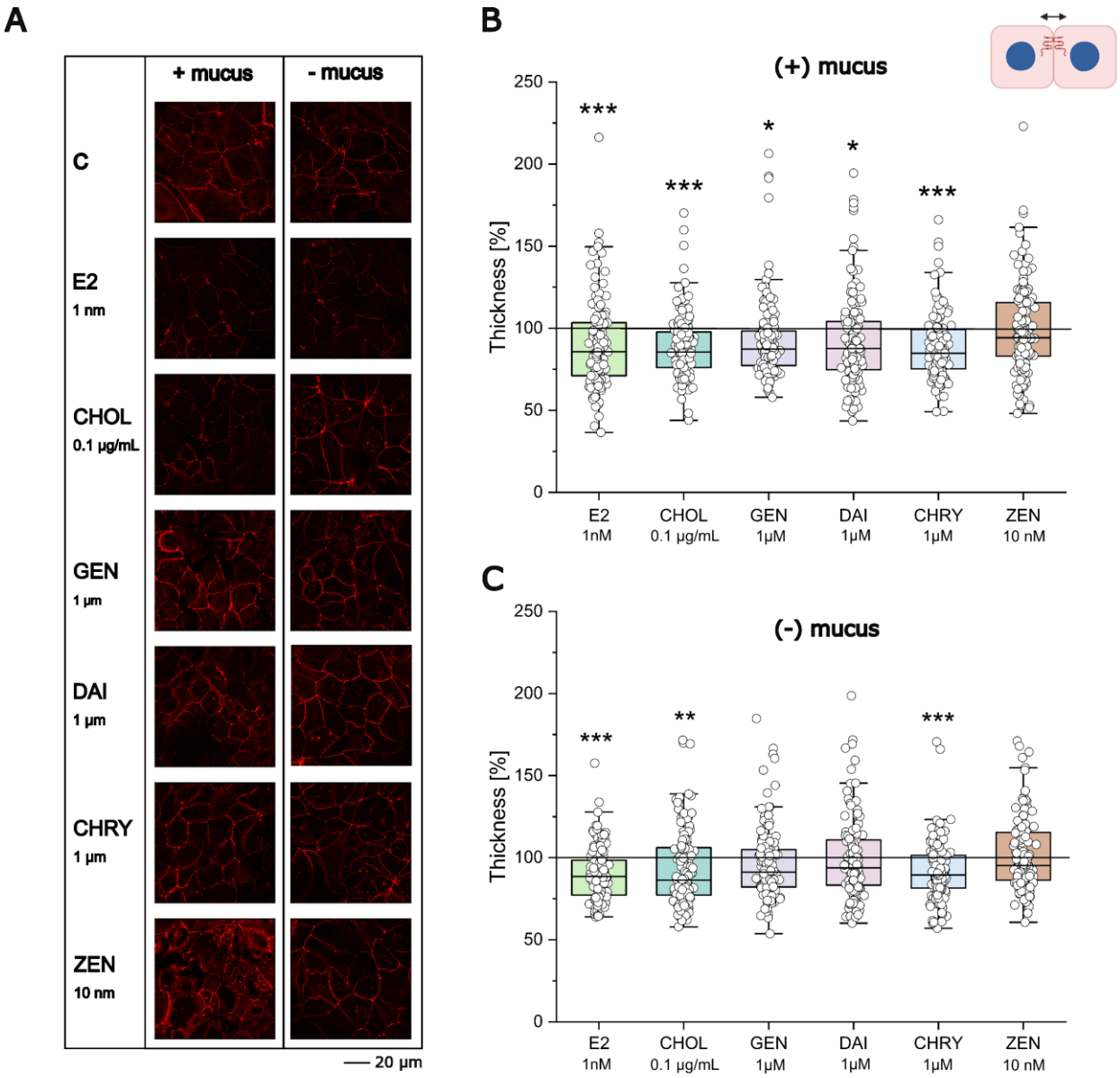


Fig 4.6 CDLN4 expression in Caco-2/HT29-MTX-E12 co-culture after treatment with EACs. (A) representative 63 X images of treated cells with nuclei in blue, CLDN4 signal in red (B,C) CDLN4 expression in Caco-2/HT29-MTX-E12 co-culture with (B) and without (C) mucus after exposure to endocrine active compounds. CLDN4 thickness signal of 15 randomly chosen tight junctions per 2D image, resulting in 135 distance measurements per condition, from 3 images per condition from 3 biological replicates, with *p < 0.05, **p < 0.01, ***p < 0.001 compared to control, Student's t-test (Welch correction); schematic representation of tight junction evaluation created with Biorender.com.

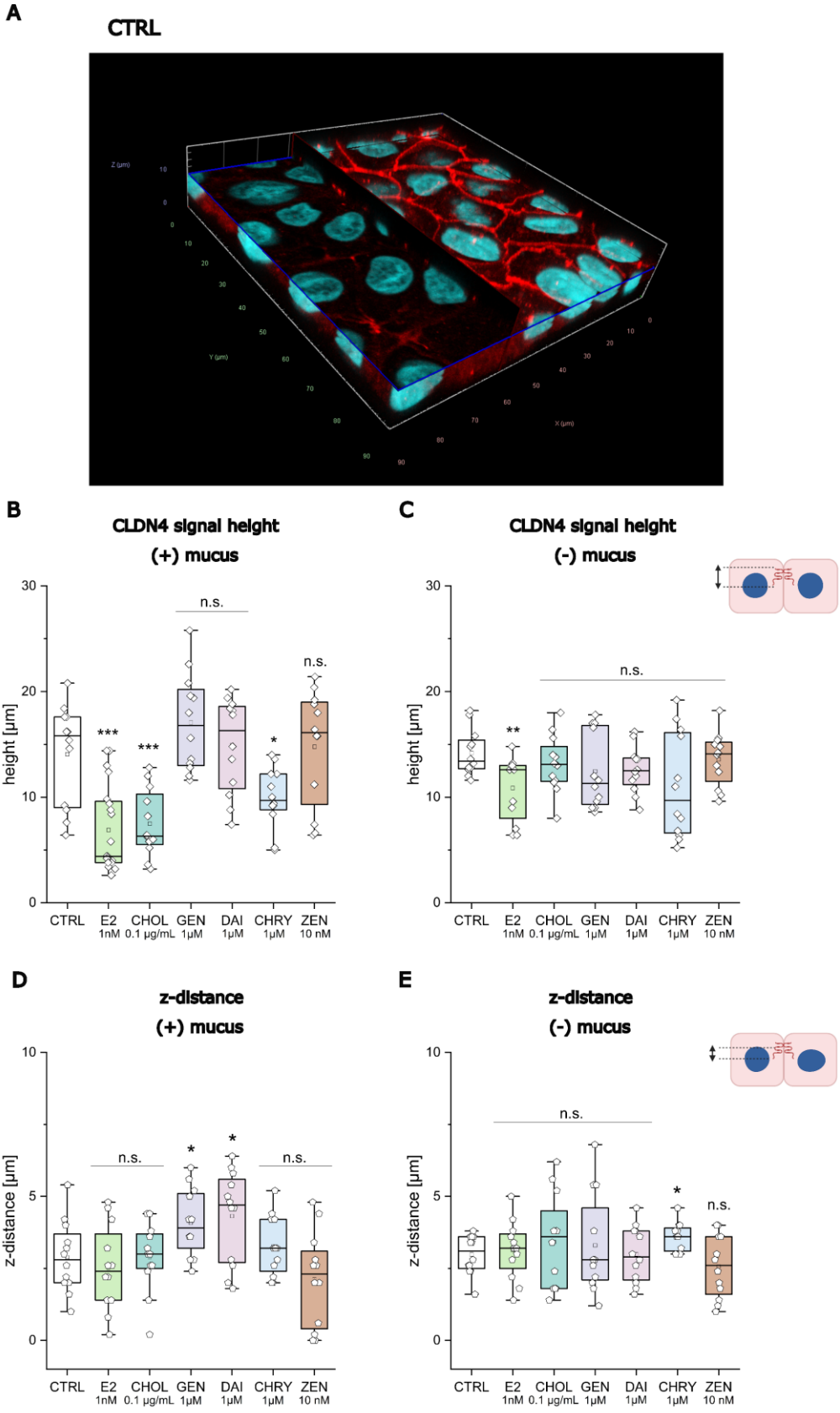


Fig 4.7 CLDN4 signal height and z-distance of CLDN4 stained Caco-2/HT29-MTX-E12 co-culture. (A) representative z-stack of control cells with CLDN4 in red and nuclei in blue (B,C) CLDN4 signal height with (B) mucus and without (C) mucus from n=3 z-stacks from 3 biological replicates (D,E) z-distance with (D) and without (E) mucus from n=3 z-stacks from 3 biological replicates, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control, Student's t-test (Welch correction); schematic representation of tight junction evaluation created with Biorender.com.

4.5 Deoxynivalenol

4.5.1 PBPK-model

For estimating the fate of DON after oral intake in comparison to the other compounds of interest, physiologically based pharmacokinetic modelling (PBPK) with concentrations and parameters as described in section 3.2.1, were applied also in this case. The model evaluated the maximum concentration C_{max} [µM] of DON in each tissue during seven consecutive days of 0.58 µg/kg bw DON intake per day. C_{max} [µM] was predicted to reach its highest value in the gut with 4.89 nM, shown in **Fig 4.8** panel A. High levels of DON are also found to be present in the liver, in comparison to that, all other tissues showed low values. Evaluating the fate of DON in the gut specifically, no accumulation throughout the 7-day exposure was predicted, as shown in **Fig 4.8** panel B.

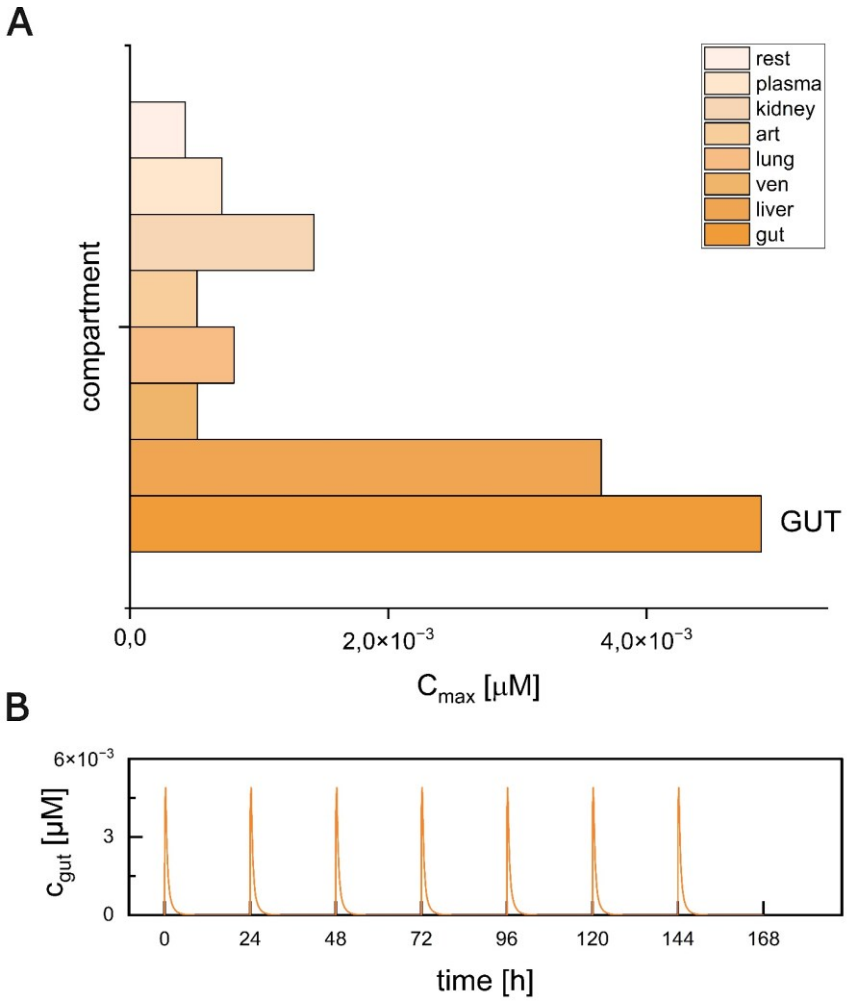


Fig 4.8 PBPK-model via ICE for oral DON exposure (0.58 µg/kg bw per day) simulation duration of seven days. (A) C_{max} [µM] of DON in gut, liver, veins, lung, artery, kidneys, plasma and rest (B) DON concentration [µM] over time in gut tissue.

4.5.2 Cytotoxicity

As from the data collected with the endocrine active chemicals, all compounds seemed to return similar effects to the TJ protein CLDN4, experiments were performed with a molecule known for its activity on the intestinal epithelium and a different molecular mechanism. This step was necessary to evaluate the morphometric adaption of the co-culture in response to a reference molecule and limit the presence of artefacts related to the quantification strategy. More specifically, DON was chosen in virtue of its previously described capacity to modulate the expression levels of TJ proteins and alter the biophysical properties of the cell membrane as well as the lipid biosynthesis machinery[73–75]. Concentration dependent cytotoxic effects of DON on Caco-2 and HT29 cells were investigated via CellTiter-Blue® assay as described in section 21. 24 h incubation with DON at concentrations of 0.1 µM, 1 µM, 2.5 µM, 5 µM and 10 µM led to significant changes in cell viability for both cell lines, as shown in **Fig 4.9** in test over control [%]. Panel A displays Caco-2 cell viability after DON exposure. After a slight increase in cell viability for 0.1 µM, a strong decrease with increasing concentration of DON is evident. 1 µM DON led to 87 % cell viability in Caco-2 cells. At 2.5 µM, 72 % of the cells were still viable in comparison to control. Cell viability nearly stagnated for higher concentrations since 5 µM and 10 µM led to 71 % and 73 %, respectively. For HT29, the onset started at 1 µM with 74 % cell viability, followed by further decrease for 2.5 µM DON with 64 %, as shown in **Fig 4.9** panel B. Incubation with DON at concentrations exceeding 2.5 µM did not lead to significantly lower cell viability but stayed at around 62 % for 5 µM and 63 % for 10 µM.

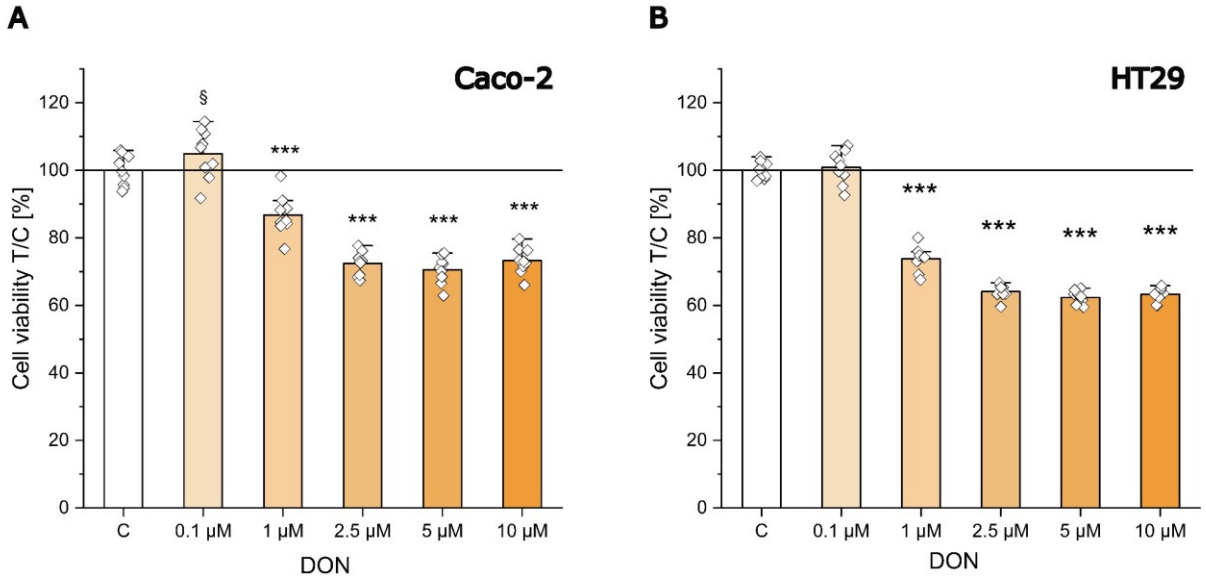


Fig 4.9 Viability of Caco-2 and HT29 cells after DON exposure. DON induces cytotoxic effects in Caco-2 (A) and HT29 (B) cells. Cell viability [%] after 24 h DON exposure of 0.1 µM, 1 µM, 2.5 µM, 5 µM, and 10 µM, data derived from n=3 biological replicates in technical triplicates, with $^{§}p < 0.05$, ***$p < 0.001$ compared to control, one-way ANOVA.

4.5.3 Membrane Fluidity

To evaluate the effects of DON on the fluidity of cell membranes, a PDA-based membrane fluidity assay was performed using Caco-2 cells. Both 10 min and 24 h exposures to DON at concentrations of 0.1 µM, 1 µM, 2.5 µM, and 10 µM were conducted and the results are shown in **Fig 4.10** as percentages relative to control [%]. For the 10 min assay, H_2O_2 (1 mM) was applied as positive control and induced a significant ($p < 0.001$) decrease in membrane fluidity, confirming the validity of the assay. 10 min exposure to DON at concentrations of 0.1 µM, 1 µM and 2.5 µM DON resulted in a considerable decrease in membrane fluidity for all three concentrations ($p < 0.01$). Palmitic acid (PA, 250 µM) served as positive control in the 24 h assay and reduced membrane fluidity to below 70 %, thereby validating the experimental setup. 24 h exposure to 0.1 µM DON significantly increased the membrane fluidity of Caco-2 cells to more than 110 % compared to controls ($p < 0.01$). Exposure to 1 µM and 2.5 µM DON showed similar effects, with membrane fluidity values of approximately 108 % relative to controls. In contrast, 10 µM DON did not induce significant effects after either 10 min or 24 h of exposure.

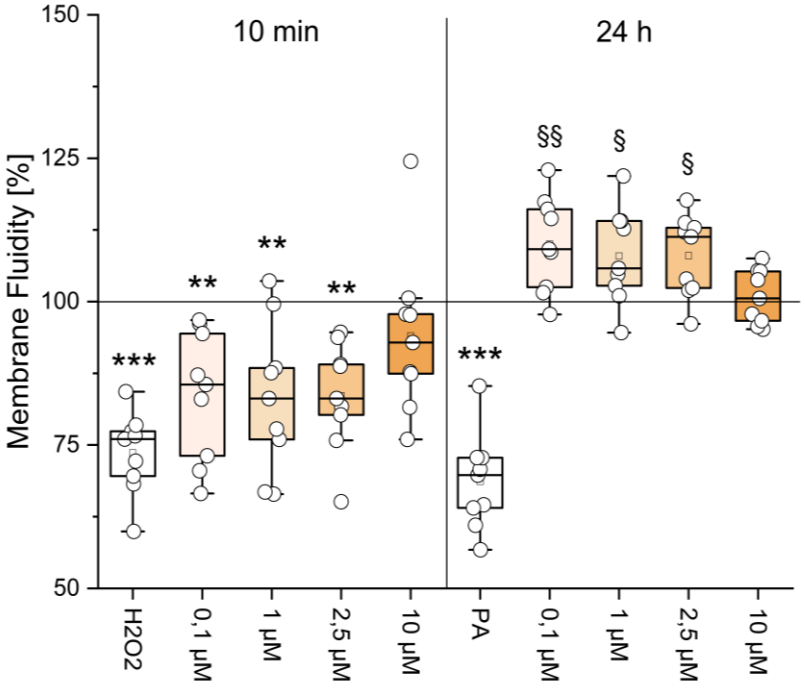


Fig 4.10 DON induces change in membrane fluidity in Caco-2 cells after 10 min and 24 h exposure. Membrane fluidity [%] after 0.1 µM, 1 µM, 2.5 µM and 10 µM DON exposure, positive controls H_2O_2 (1 mM) and PA (250 µM), data derived from n=3 biological replicates in technical triplicates, with */§ $p < 0.05$, **/$^{§§}p < 0.01$, ***/$^{§§§}p < 0.001$ compared to control, one-way ANOVA, In the 10 min assay, DON was tested in parallel to substances presented in figure 4.4. The same positive controls were used and presented in both graphs.

4.5.4 Immunofluorescence

4.5.4.1 Nuclei Analysis

The Caco-2/HT29-MTX-E12 co-culture was tested on morphological changes in the nuclei following 24 h DON exposure (2.5 µM). Relative area, aspect ratio, circularity, roundness and solidity of the nuclei were assessed and are presented in **Fig 4.11** (panel A and B). Significant increase in relative area was caused by DON exposure in the co-culture with and without mucus compared to control (panel C). Panel D shows the nuclei solidity, which was increased following DON treatment under mucus reduced conditions ($p < 0.05$). No significant changes in aspect ratio, circularity and roundness induced by DON were observed.

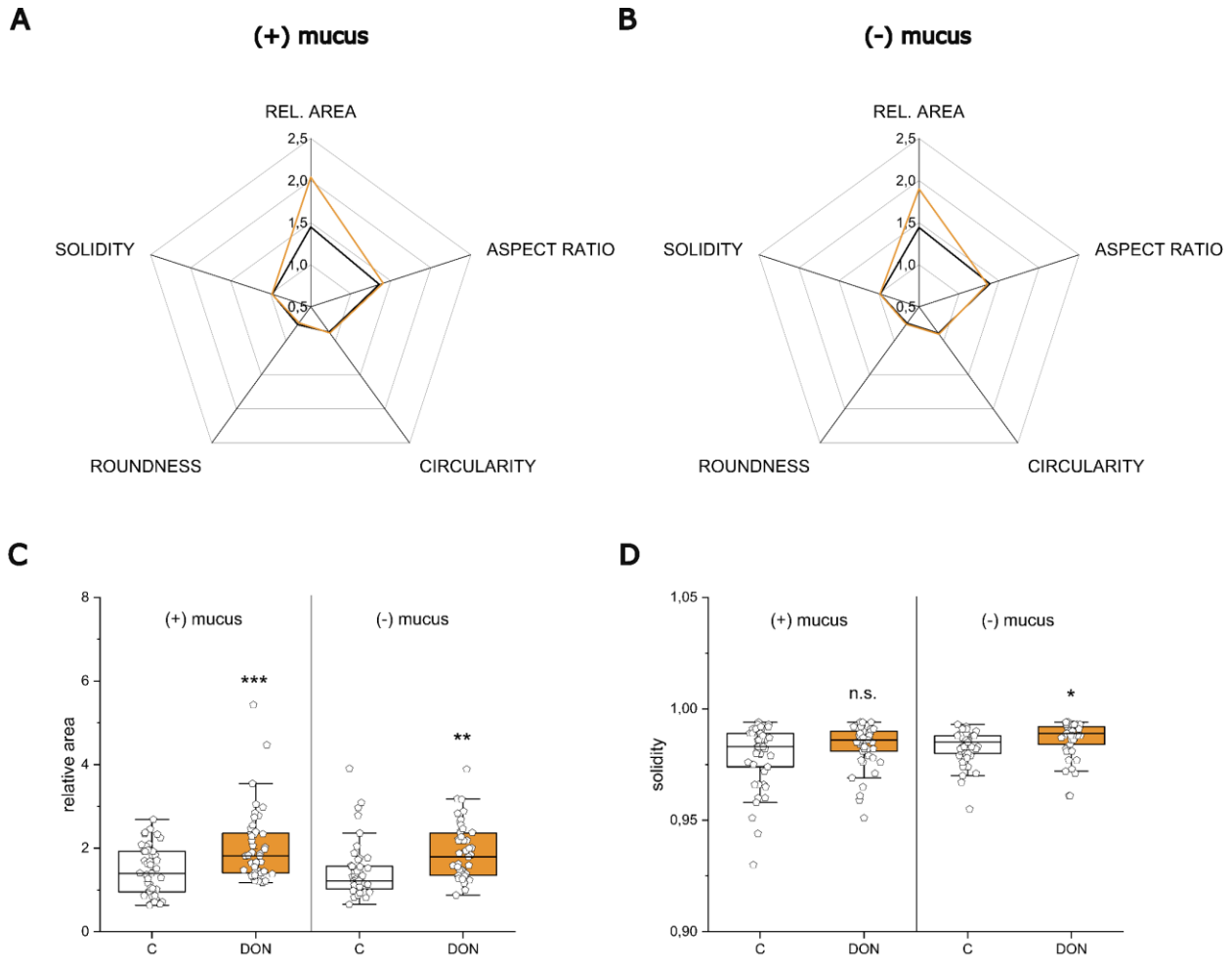


Fig 4.11 Nuclei morphology of Caco-2/HT29-MTX-E12 co-culture treated with DON (2.5 µM). (A,B) Morphological nuclear descriptors relative area, aspect ratio, circularity, roundness, solidity of nuclei in co-culture (A) with mucus (B) without mucus; (C) relative area of nuclei with and without mucus, data derived from n = 45 nuclei from n=3 biological replicates, with **$p < 0.01$, ***$p < 0.001$, Student's t-test (Welch correction); (D) nuclei solidity with and without mucus, data derived from n=45 nuclei from 3 biological replicates with *$p < 0.05$, Student's t-test (Welch correction).

4.5.4.2 CLDN4 expression

The expression of tight junction protein CLDN4 strongly correlates with barrier integrity[55,69,70] and is therefore of high relevance for the present study. To assess the expression and localization of the tight junction protein of interest, CLDN4 thickness, the z-distance and the CDLN4 signal height were quantified.

Representative 2-D images of the DON exposed co-culture as well as the control with both mucus conditions are illustrated in **Fig 4.12** panel A, CLDN4 tight junctions are marked in red. As shown in **Fig 4.12** panel B, CLDN4 thickness was significantly increased upon DON exposure (2.5 µM). In the co-culture with mucus, DON induced an increase of approximately 8 % in comparison to control ($p < 0.01$). After mucus reduction, an increase of 5 % in average CLDN4 thickness was caused by exposure to DON ($p < 0.05$).

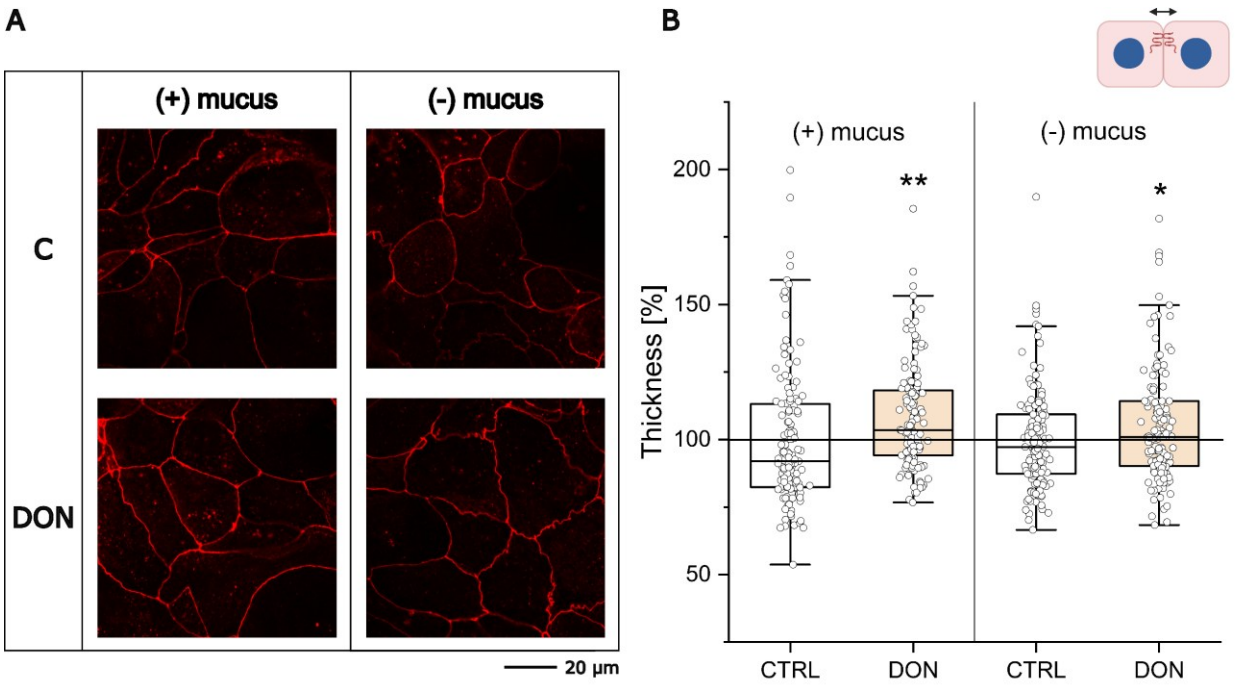


Fig 4.12 CLDN4 expression after 24 h DON exposure (2.5 µM) in Caco-2/HT29-MTX-E12 co-culture. (A) Representative 63X images of DON exposed co-culture and controls with and without mucus (B) CLDN4 Thickness [%] with and without mucus, data derived from n = 135 distance measurements per condition from 3 biological replicates, with *$p < 0.05$, **$p < 0.01$ compared to control, Student's t-test (Welch correction); schematic representation of tight junction evaluation created with Biorender.com.

To estimate the distribution of CLDN4 and the overall organization of the cell monolayer, the CDLN4 signal height [µM] was quantified (**Fig 4.13**, panel A). A slight increase in CDLN4 signal height was observed upon DON exposure for the cell model with mucus ($p < 0.05$). Under mucus reduced conditions, no significant changes in the CDLN4 signal height were tested.

The z-distance, illustrated in **Fig 4.13** panel B, was defined as the difference along the z-axis between CLDN4 tight junctions and the nuclei. Exposure to DON did not induce any significant changes in z-distance in co-cultures with an intact mucus layer. In contrast, a reduced z-distance was observed when mucus was removed prior to DON exposure.

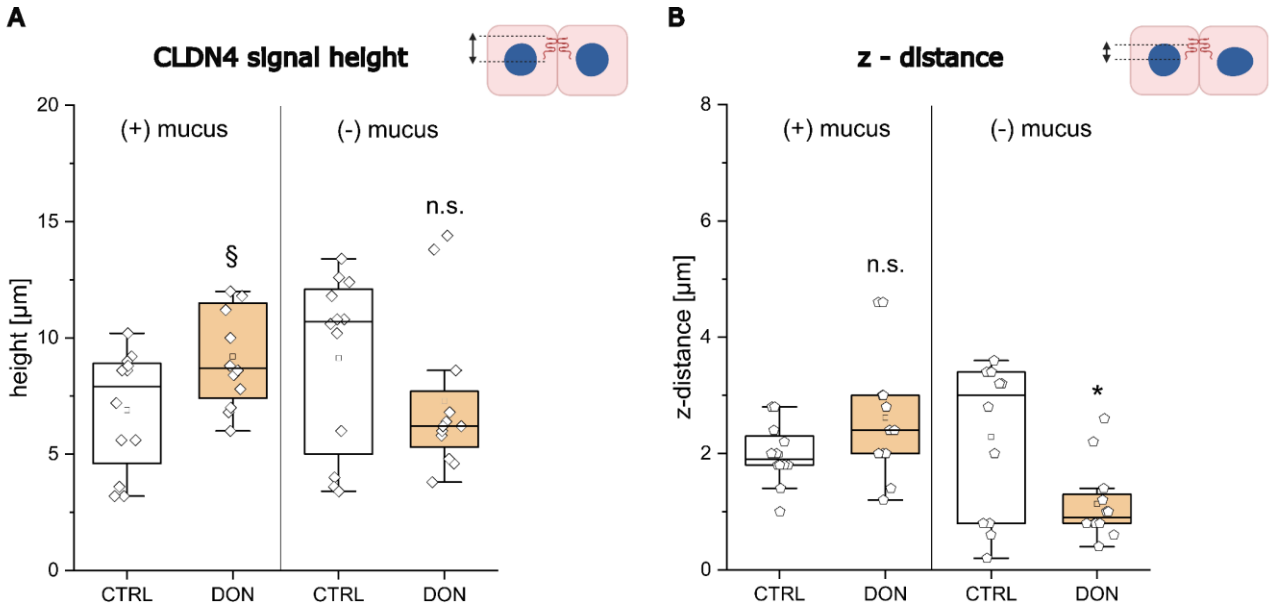


Fig 4.13 CLDN4 signal height and z-distance of CLDN4 stained Caco-2/HT29-MTX-E12 co-culture after DON (2.5 µM) exposure. (A) CDLN4 signal height with and without mucus from n=3 z-stacks from n=3 biological replicates, $^{§}p < 0.05$ compared to control, Student's t-test (Welch correction); (D) z-distance with and without mucus from n=3 z-stacks from 3 biological replicates $*p < 0.05$ compared to control, Student's t-test (Welch correction); schematic representation of tight junction evaluation created with Biorender.com.

5 Discussion

The barrier integrity of the intestinal epithelium is essential for the protection against foodborne pathogens, toxins and antigens[4]. Insufficient intestinal barrier function facilitates the unregulated entry of ingested compounds into the bowel wall which further promotes the development of various inflammatory diseases, including Crohn's disease, ulcerative colitis and colon carcinoma[4]. Therefore, an efficiently functioning intestinal barrier is an indispensable health determining factor. Tight junctions (TJs) play an essential role for intestinal barrier integrity and contribute to the regulation of paracellular transport[53]. TJ expression and assembly can be influenced by various dietary compounds, including phytoestrogens, amino acids and vitamins[76]. Phytoestrogens are naturally occurring plant secondary metabolites with endocrine activity and are known for their positive health effects.[41] Evidence indicates that the expression and/or assembly of TJs in intestinal cells is supported by several phytoestrogens such as quercetin[77], naringenin[78] and kaempferol[79].

In the current study, the effect on barrier integrity was further investigated for the phytoestrogens genistein, daidzein and chrysin. Their influence on the expression of tight junction protein CLDN4 was observed via *in vitro* tests in Caco-2/HT29-MTX-E12 co-culture. The applied phytoestrogens showed no cytotoxic effects in cultures of Caco-2 and HT29 in the concentrations relevant for the study (0.1-10 µM), tested via CellTiter-Blue® assays. Even if the data could not be verified with an independent method in the frame of this work, according to a 7-day simulation with pharmacokinetic modelling (PBPK) via ICE, relatively high concentrations in the gut are expected for GEN and DAI, in contrast to CHRY which leads to moderate to low concentrations in the gut. None of the tested phytoestrogens are estimated to accumulate in the gut tissue. Prior to examining TJs expression, potential changes in membrane fluidity due to exposure with the compounds of interest were tested in Caco-2 culture. This approach is based on recent research, suggesting that alterations in membrane fluidity can affect membrane protein function[18,19]. Incubation time of 10 min and 24 h were applied, to assess their effects on the membrane fluidity in different time scales. H_2O_2 was used as positive control for all 10 min exposure membrane fluidity assays conducted in this study. The significant reduction of membrane fluidity due to H_2O_2 validated the accuracy of the experiment. Increased membrane rigidity was previously observed upon exposure to PA (≥ 25 µM) for 24 h[68]. This could be confirmed in the conducted 24 h experiments, supporting the reproducibility of the presented results. Regarding the phytoestrogens, genistein induced a decrease in membrane fluidity after 10 min, which restored to values comparable to controls after 24 h exposure. While daidzein showed no relevant effects on membrane fluidity, the phytoestrogen chrysin led to enhanced fluidity in the membrane after 24h exposure.

In this study, immunofluorescence assays enabled a detailed examination of the expression and localization of the CLDN4 tight junction protein, structural rearrangements of the cell monolayer and morphological parameters of the nuclei. To mimic the intestinal gut epithelium with corresponding mucus layer, a co-culture consisting of Caco-2 and goblet cell-like HT29-MTX-E12 was implemented, in which the cells are polarized after a 7-day differentiation period. In addition to experiments on the co-culture with mucus layer, mucus removal was performed to assess the effects under mucus reduced conditions which potentially occur through diseases like the irritable bowel syndrome.

24 h exposure of the co-culture with genistein (1 µM) led to a significant increase in relative area of the nuclei, which could not be observed at mucus reduced conditions. Changes in nuclei morphology are of particular interest since alterations in nuclei size and shape are not only present during differentiation and development of cells but are also frequently observed during the progression of diseases, including cancer[80]. Interestingly, genistein also induced a decrease in CLDN4 tight junction thickness combined with an increase in z-distance for the model with mucus, whereas no significant effects could be seen in the co-culture without mucus. Similar observations were made for the phytoestrogen daidzein (1 µM), which led to a reduction in CLDN4 TJ thickness with intact mucus layer only. The z-distance between CLDN4 and the center of the nuclei was also decreased in the co-culture with mucus layer. For the co-culture without mucus no significant changes could be observed in the immunofluorescence assays conducted in the present work. According to this study, chrysin (1 µM) significantly reduces tight junction thickness of CLDN4 in Caco-2/HT29-MTX-E12 co-culture both with and without mucus. These effects align with observations by Noda et al.[81] who described a decreased total CLDN4 expression in Caco-2 cells upon 24 h chrysin exposure (100 µM), tested via immunoblot analysis. Noda et al. also demonstrated adverse effects on TJ permeability, seen in altered transepithelial electrical resistance (TEER).[81] In this work, chrysin led to a decreased CDLN4 signal height in the co-culture with mucus, indicating a slight change in the structural arrangement of the monolayer. After mucus removal, chrysin induced an increase in nuclei circularity as well as in the z-distance between CLDN4 and the nuclei.

Natural endocrine active substances do not only derive from the plant kingdom in form of phytoestrogens but can also be produced by fungi, such as the mycotoxin ZEN. There is evidence that ZEN does influence intestinal barrier integrity, which makes it an interesting compound for the present research question. Studies by Huangfu and colleagues demonstrated a decreased TEER and negative effects on TJ protein CLDN4 in Caco-2 cells after exposure with 20 µM ZEN for 24 h.[82] However, the applied concentration of 20 µM seems high in comparison to toxins levels which can be typically expected in the gut, as described in section 2.4.1, and data has to be interpreted with caution. In this

study, the effects of ZEN at concentrations in the nanomolar range (10 nM) were tested in order to investigate potential effects at low but hormonally active concentrations. The endocrine modulating activity for 10 nM ZEN was proven previously also in our department as published by Grgic et.al.[47]. In the present study, no cytotoxic effects in Caco-2 and HT29 were observed for 1-100 nM ZEN via CellTiter-Blue® assay. PBPK modelling indicated that ZEN is mainly present in the gut (C_{max} 0.89 nM) in comparison to other tissues after oral administration but does not accumulate in the aforementioned tissue during the 7-day simulation. Further, *in vitro* PDA assays with Caco-2 culture revealed that ZEN has the potential to alter membrane fluidity. While no changes were observed after 24 h incubation with the mycoestrogen, the "short term" exposure of 10 min induced a significant decrease in membrane fluidity. In the co-culture Caco-2/HT29-MTX-E12 with mucus layer, the 24h exposure with ZEN (10 nM) increased the relative area of the nuclei, which could not be observed in the mucus reduced model. Immunofluorescence assays revealed that the concentration of 10 nM ZEN leads to no significant effects on the tight junction protein CLDN4 and on the structure of the monolayer.

The experiments conducted on natural endocrine active substances were also done with the endogenous substances E2 and CHOL as reference. Since oral intake of the compounds is plausible through diet or therapy, their fate after ingestion is important when studying intestinal barrier integrity. Predictions via PBPK-ICE showed that the concentration of E2 after oral intake is expected to be low in relation to other tissues. In contrast, CHOL is predicted to be mainly present in the gut after oral intake. According to the 7-day simulation, daily oral administration leads to accumulation of both endogenous substances in the gut tissue, reaching approximately 0.5 nM for E2 and 102 µM for CHOL at the endpoint. These values lie below cytotoxic levels, since incubation of Caco-2 and HT29 with 0.1 - 10 nM E2 and 0.01-1 µg/mL CHOL led to no adverse effects on cell viability in the CellTiter-Blue® Assay. The membrane fluidity of Caco-2 cells was significantly altered by the steroid hormone E2 (1 nM), leading to a decrease after 10 min and an increase after 24 h exposure. CHOL (0.1 µg/mL) indicated increased fluidity of the membrane after 10 min incubation but did not show any effect after 24 h for the applied concentration. Nuclei analysis of the co-culture with intact mucus layer revealed a decreased nuclei solidity and increased aspect ratio due to 24 h exposure with CHOL. After mucus removal, E2 induced an increased nuclei solidity, circularity and roundness. According to immunofluorescence studies, both substances seem to downregulate expression of CLDN4. Incubation of the co-culture with E2 and CHOL led to a significant decrease in tight junction thickness of CLDN4, both with mucus layer and with reduced mucus. Similar findings were done for the tight junction protein ZO-1 by Zhou et. al.[83], who reported a decreased ZO-1 expression after exposure to E2 in Caco-2 cells. In the present study, the adverse effects on tight junction thickness were accompanied by a decreased CDLN4 signal height for both compounds

in the co-culture with mucus. E2 also decreased the height of the CLDN4 signal after reduction of the mucus.

As part of the study, the effect of the mycotoxin DON on intestinal barrier integrity was tested in addition. DON does not show endocrine activity[48] but is a potent inhibitor of protein synthesis[49]. Since intestinal epithelium cells are constantly renewed, a potential impact of DON on barrier integrity due to its protein synthesis inhibitory properties appears plausible. In a 21-day differentiated Caco-2 cell culture DON (5000 ng/ml) was shown to reduce expression of the protein CLDN4, accompanied with increased cell layer permeability.[84] In this study, effects of DON are observed for a Caco-2/HT29-MTX-E12 co-culture after 7 days differentiation time. Cytotoxic effects of DON were observed in Caco-2 and HT29 cells monocultures with a dose dependent decrease in cell viability for both cell lines. DON induced cytotoxic effects starting with 1 µM. Cell viability for 2.5 µM DON reached 72 % and 64 % in Caco-2 and HT29, respectively. A point to consider, since the TDI for DON is set on 1 µg/kg bw per day[31], which theoretically results in 2.25 µM DON in the small intestine directly after oral intake, assuming an intestinal volume of 105 mL under fasting conditions[22]. DON showed effective changes in membrane fluidity of Caco-2 cells in the PDA based assay. DON exposure of 0.1 µM, 1 µM and 2.5 µM led to a significant decrease in membrane fluidity after 10 min and to a significant increase after 24 h. 10 µM did not induce any statistically relevant effects in membrane fluidity in the present experiments. Interestingly, 24 h exposure with DON had a strong impact on the relative area of the nuclei in the Caco-2/HT29-MTX-E12 co-culture. The compound of interest led to an increase in relative area in presence and absence of mucus. After mucus removal, DON exposure also led to a slight increase in nuclei solidity. In this study, 24 h exposure of DON (2.5 µM) induced a significant increase in CLDN4 tight junction thickness in both cell models (+/- mucus). The CDLN4 signal height was slightly increased for the co-culture with mucus and the z-distance between CLDN4 and center of the nuclei was decreased for the mucus reduced model. Comparable observations were done by Beisl J and colleagues[85], who observed an increased CLDN4 signal of 127 ± 29 % after 24 h incubation with DON (10 µg/mL, 33.74 µM) in Caco-2[85]. In the cited study, also TEER values were increased upon 24h treatment of Caco-2 with 1µg/mL (3.37 µM) and 10 µg/mL DON[85]. Groestlinger J et al. showed an enhancing effect of DON on TEER in Caco-2 cultures at equivalent concentrations as in the present study (2.5 µM) after 24 h exposure, which was followed by a significant decrease after 48 h exposure[86]. Different effects in dependency on proliferation and differentiation status of Caco-2 were described by Wang et. al.[87], who states that DON (5 µM) reduced CLDN4 expression during proliferation after 3 days and in the differentiated cell model after 21 days. In contrast, DON treatment increased the TEER as well as CLDN4 expression after 11 days of differentiation time of the culture.[87] Other studies also reported a decrease in CLDN4 expression, which can be explained due to longer differentiation time (17-19 days)[88] or different cell lines such as IPEC-1[89].

In vivo, DON showed chronic and acute toxic effects on humans and animals[31,90]. For example, studies on pigs led to hampered morphology of the gastrointestinal tract such as villi fusion and decreased CLDN4 expression levels in jejunum and ileum due to DON contaminated feed[90]. The variable effects of DON in dependence on the cell model and the differences of *in vitro* and *in vivo* results indicate potential challenges in standardization when developing new approach methodologies (NAMs)[91].

6 Conclusion

The focus of this thesis was to investigate the effects of natural endocrine active substances on intestinal barrier integrity via *in vitro* assays. The study elucidated the impact of foodborne estrogenic compounds GEN, DAI, CHRY and ZEN on the expression of tight junction protein CDLN4, an important constituent in the intestinal epithelium. The experiments were supported by additionally testing the endogenous substances E2 and CHOL and the non-hormonally active mycotoxin DON. The observations revealed notable changes in CLDN4 organization induced by the test substances, pointing out the need for further investigations in this field.

Based on NAMs[91] methodologies, in this work monocultures and co-cultures cell models were combined to characterize the effects of compounds of natural origin sharing similar structural features. The development of NAMs is an important step towards the vision of 3R (replacement, reduction and refinement) proposed by Russell and Burch[92], however, the complexity of biological systems presents challenges for standardization. To conclude, the observations made in this thesis contribute to closing knowledge gaps regarding the effects of natural endocrine active substances on the intestinal epithelium.

Supplementary

Name	Supplier	Reference ID	Additional Information
Triton-X 100	SIGMA	514510	
CellTiter-Blue® solution	ROTH	270298309	
PBS-A	Self-Prepared	ROTH 3904.1 VMR 26764.298 ROTH 0962.2 ROTH 4984.1	0.4 g KH_2PO_4; 0.4 g KCl; 16 g NaCl; 4.4 g Na_2HPO_4; dissolved in autoclaved H_2O (1L), pH 7.2; membrane-filter (Filtropur SARSTEDT 500 mL 0.2 µM REF: 83.39.41.001)
Permeabilization Buffer	Self-Prepared		0.2 % Triton-X in PBS-A
Washing buffer	Self-Prepared		0.05 % Triton-X in PBS-A
NES (normal external solution)	Self-Prepared		2.8 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 10 mM Hepes, 2 mM $MgCl_2$, 10 mM glucose (pH 7.35)
Roti-Mount Flourcare + DAPI	ROTH	HP 20.1	
Dulbecco's Modified Eagle Medium	gibco	21063-029	
Donkey Serum	Sigma	D9663-10ML	
Fetal calf serum	gibco	10270-106	
Trypsin	gibco		
Claudin 4 Monoclonal Antibody (3E2C1), Alexa Fluor™ 594	Invitrogen	329194	
N-Acetyl-L-cysteine	Merck	1.12422.0025	
Pyrene decanoic acid	abcam	Ab274309	

Tab 0.1 Chemicals

Name	Supplier	Reference ID	CAS Number
17-beta-estradiol	Sigma	200-023-8	50-28-2
Cholesterol	Sigma	C4951	57-88-5
Genistein	Extrasynthese	1372 S	446-72-0
Daidzein	Extrasynthese	1370 S	486-66-8
Chrysin	Thermo Fisher Scientific	C80105-25G	480-40-0
Zearalenone	Sigma	50-177-8736	17924-92-4

Tab 0.2 Test substances

Cell line	Company	Additional Information
Caco-2 cells	Sigma-Aldrich Corporation (St. Louis, USA)	Kindly provided by Prof. Marko
HT29-MTX-E12 cells	Sigma-Aldrich Corporation (St. Louis, USA)	Kindly provided by Prof. Marko
HT29 cells	Sigma-Aldrich Corporation (St. Louis, USA)	Kindly provided by Prof. Marko

Tab 0.3 Cell cultures

Instrument	Company	Product number
Neubauer counting chamber	Paul Marienfeld GmbH & Co. KG (GER)	640110
Pasteur pipette 150 mm	SARSTEDT	15.310.965
Pipette tips 10–5000 µL	SARSTEDT	703.060
Serological pipette 10 mL, sterile	SARSTEDT	861.254.001
Solution basin 5 mL / 25 mL	Biologix Europe GmbH	25-1025 / 25-0051 / 25-1100
8-well ibidi µ-Slide	Ibidi	80826
Gloves; nitrile, powder-free, blue	Th. Geyer GmbH & Co. KG	7.696.900
Microtubes (1 mL / 1.5 mL / 2 mL)	SARSTEDT	72.706
Centrifugation tubes 15 mL	SARSTEDT	62.554.016
Centrifugation tubes 50 mL	SARSTEDT	72.691.001
Costar 96-well plate black	Corning incorporated	3603
TC-Flask T175 Standard, vent cap	SARSTEDT	833.911.002
TC-Flask T75 Standard, vent cap	SARSTEDT	833.911.002
TC-Plate 96-well Standard, F	SARSTEDT	833.924

Tab 0.4 Consumables

Instrument	Specification	Company
Freezer	Temperature: -20°C	Liebherr-Hausgeräte GmbH
Fridge	Temperature: 4°C	Liebherr-Hausgeräte GmbH
Heating plate	MS-H-PRO+	DLAB Scientific Co
Incubator	Heracell 240i CO_2	ThermoFisher
Laminar flow cabinet	CytoFAST Elite	FASTER
Microplate reader	Synergy H1	BioTek
Microscope	CKX53	Olympus
Multichannel pipette	30–300 µL	Eppendorf SE
Pipettes	10, 20, 100, 200, 1000, 5000 µL	Eppendorf SE
Vortex shaker	Lab dancer	IKA
Water bath	GD100	Grant instruments Ltd.
Fridge	Temperature: 4°C	Liebherr
Freezer	Temperature: -20°C	Liebherr
Zeiss LSM710	Elyra PS. 1	Carl Zeiss AG

Tab 0.5 Instruments (WÄ: Währingerstr. 38 Chemistry faculty; Group Doris Marko; UZA2 Pharmazie Geozentrum LMC Chemistry faculty; Group Giorgia Del Favero)

Software	Version	Company
Excel	202505	Microsoft Corporation
Gen5		Biotek Instruments Inc.
OriginPro®	2025	OriginLab Corporation
ImageJ (FIJI)	2.16.0	Wayne Rasband, National Institutes of Health
ZEN (black)	2012 SP5 FP3 (14.0.20.201)	Carl Zeiss AG
Inkscape	1.4.2	Inkscape Community

Tab 0.6 Software

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