Apparent permeability of enniatins in *in vitro* intestinal model

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MSc thesis

By Pooja Khadka

Submitted to Toxicology department of Wageningen University and Research.

Supervised by Jingxuan Wang and

Nynke Kramer

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WAGENINGEN
UNIVERSITY & RESEARCH

Abstract

Enniatins are a secondary metabolite produced by *Fusarium species*. They are an emerging mycotoxin and has several proven immunotoxicity cytotoxicity in *in vitro*. Besides, it also has antibacterial, antifungal, herbicidal, and insecticidal effects. Moreover, increasing climate change has increased the presence of enniatins in feed and foods that rises the discussion of enniatins. Very few studies have been done regarding the transport of enniatins and there has been no study has been done to this date on the apparent permeability of enniatins. Apparent permeability (P_{app}) is an important parameter that is used in physiologically based (PBK) models. This model is used to estimate the kinetics of chemical which is helpful in assessing the risk assessment. This study aimed to compare P_{app} of the four ENNs (1uM ENNA, 1uM ENNA1, 1uM ENNB, and 1uM ENNB1) in the Caco-2 permeability assay for input into PBK models. The transport study was conducted in Caco-2 differentiated cell in 12-transwell and 24-transwell at timepoint of 30, 60, 120 and 180 minutes at 37°C, 5% Co_2. The results showed that P_{app} increases with the increase of cumulative permeated amount. In 12-well, ENNB1 exhibited high permeability followed by ENNB, ENNA and ENNA1. ENNB1 P_{app} was 2, 4, and 13 times higher than ENNB, ENNA and ENNA1 respectively. In 24-transwell, P_{app} was highest for ENNA followed by ENNB1 and ENNA1. ENNA1 P_{app} was 2, 2, and 1.5 times higher than ENNA, ENNB and ENNB1 respectively. Nevertheless, P_{app} exhibited from this study shows similarities for ENNA and ENNA1 in both 12-transwell than in 24-transwell except for ENNB and ENNB1 P_{app}. Moreover, P_{app} values of ENNs in this study range from 0.43 ± 0.01 - 5.53 ± 0.98 * 10^{-6} cm/s in 12-transwell and 0.63±0.01 - 1.11± 0.03 * 10^{-6} cm/s in 24-transwell indicating ENNs as low permeable compounds.

Table of Contents

1. Introduction

1.1 Small intestine

The small intestine is the largest component of the gastrointestinal tract where digestion and absorption of nutrients, water and electrolytes occur. It consists of four different layers as Serosa, Muscularis, Submucosa, and Mucosa (DeSesso & Jacobson, 2001). The mucosa is the innermost layer that comes into direct contact with the digested food. The mucosa consists of three layers as epithelium, lamina propria, and muscularis mucosae Figure 1. The epithelium is the inner most layer of mucosa providing a barrier against harmful substances where most absorption, digestion and secretion occur (Doherty & Charman, 2002).

The intestinal epithelium consists of several key components such as cells types, epithelial barrier, microvilli, mucus layer, immune cells, permeability, and regeneration (Kong et al., 2018). The epithelium consists of cells such as enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. Enterocytes are responsible for absorbing the nutrients (e.g., ions, water, sugar, peptides, and lipids), and secreting digestive enzymes and mucus. Goblet cells are responsible for secretion of mucus which helps protect the intestinal lining and lubricates the passage of stool. This mucus layer acts as a physical barrier and also contains antimicrobial molecules. Paneth cells are responsible for secretion of antimicrobial peptides and enzymes that play a role in the body's immune defense and protection against pathogens. Enteroendocrine cells are responsible for secretion of hormones that regulate various digestive and metabolic processes. Microfold cells are specialized cells found in the follicle-associated epithelium (Peyer's patches[1]) of the small intestine. They play a role in immune surveillance by transporting antigens from the gut lumen to immune cells in underlying tissues (Kong et al., 2018).

The intestinal epithelium forms a physical barrier between the contents of the gastrointestinal tract and the body's internal environment. This is regulated by the tight junctions between enterocytes preventing the entry of harmful pathogens

[1] Peyer's patches are the components of gut-associated lymphoid tissue (GALT) in the small intestine that plays the important role in the immune surveillance of the intestinal lumen and in facilitating production of immune response within the mucosa (Jung et al., 2010; Wikipedia contributors, 2023).

and toxins while allowing the selective absorption of nutrients. Tight junctions between cells play a crucial role in controlling permeability. The luminal surface of enterocytes is covered with microvilli, tiny finger-like projections that greatly increase the surface area for nutrient absorption. Microvilli contain transport proteins and enzymes that facilitate the uptake of nutrients. The intestinal epithelium is closely associated with the gut-associated lymphoid tissue (GALT), which includes immune cells like lymphocytes and dendritic cells. These cells help monitor and respond to potential threats in the gut, contributing to gut-associated immune defense. The intestinal epithelium has a high rate of turnover and regeneration. New epithelial cells are continuously produced from stem cells located in small crypts within the epithelium (Kong et al., 2018; Fedi et al., 2021).

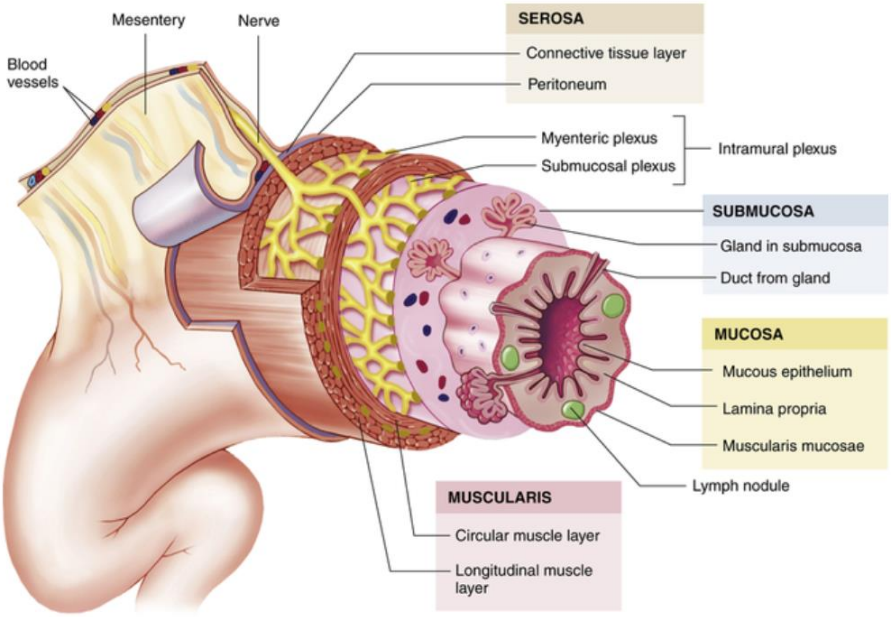

Figure 1. Anatomy of small intestine's layers (Themes, 2016)

1.1.1 Absorption mechanism in the small intestine

The absorption of drugs via the oral route is under examination through bioavailability, and good bioavailability indicates membrane via a diversity of mechanism that comprise of passive diffusion or active transport, Figure 2(Groschwitz & Hogan, 2009).

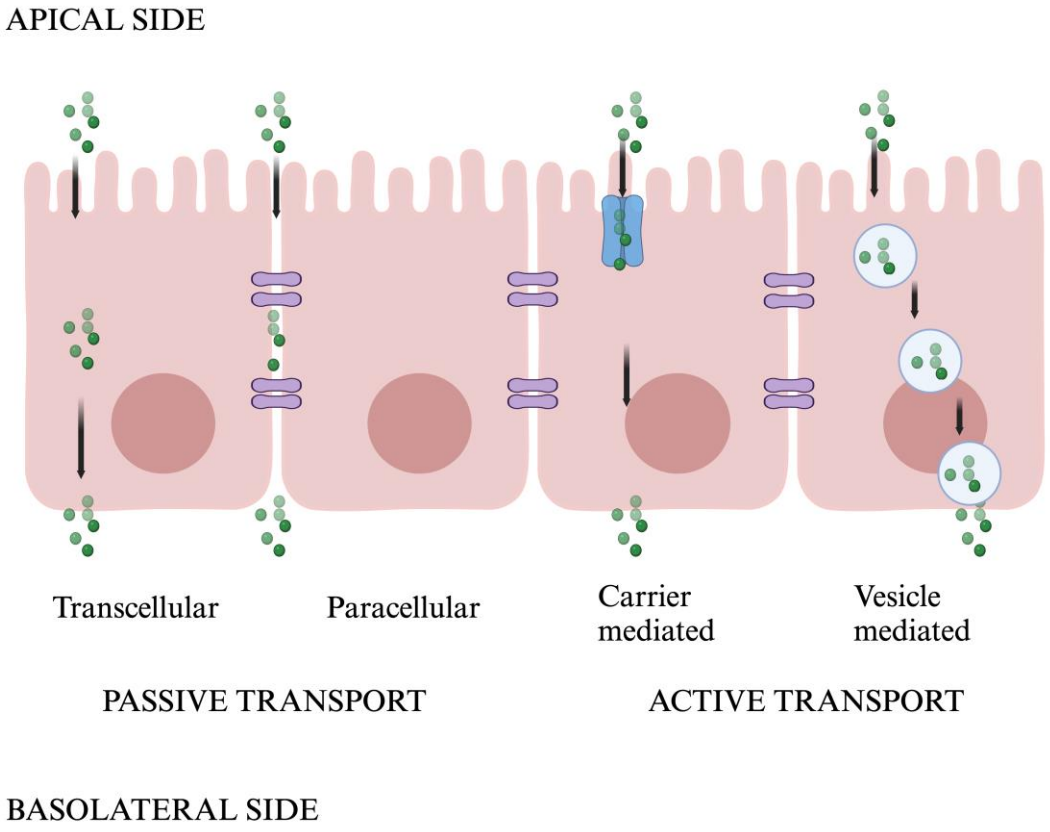

Figure 2. Transport of xenobiotic from apical to basal compartment via passive or/and active transport.

Passive diffusion is mediated by two pathways: the transcellular and paracellular pathway. The transcellular pathway involves the movement of substances directly through individual cells through the channel or carrier (Picci et al., 2022). The transcellular pathway involves absorption, transport across the cytoplasm, extrusion and release into the extracellular space. The absorption of substances occurs from luminal into cytoplasm of the cell. Transport across the cytoplasm refers to the movement of substances through the cytoplasm. Depending on the substance and its properties, this may require the involvement of specific transport proteins or undergo metabolic transformations within the cell. After traversing the cytoplasm, the substance is then transported across the opposite side of the cell as referred as extrusion. Finally, the substance is released from the cell into the extracellular space on the opposite side of the barrier. It can then enter the bloodstream, lymphatic system, or other relevant physiological compartments, depending on the location of the barrier. The paracellular pathway is involved in the transport of small molecules, ions, and solutes between adjacent

cells, primarily across epithelial and endothelial cell layers that form biological barriers. In the paracellular pathway, substances move through the intercellular spaces or gaps between neighboring cells (junction) (Groschwitz & Hogan, 2009).

The active transport pathway is mediated by the transporters that is associated to influx and efflux. Epithelial membrane transporters are classified into two superfamilies named as solute carrier (SLC) and ATP-binding cassette (ABC) transporter. These transporters are expressed on the apical and basolateral compartments of the intestine epithelium. ABC transport system requires large amount of energy, while SLC transport system doesn't require energy (Estudante et al., 2013).

ABC family includes transporters such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP 1, MRP 2, MRP 3, MRP 4, MRP 5) and breast cancer resistance protein (BCRP). P-gp, MRP 2 and BCRP transporters are efflux transporters and are highly expressed on the apical compartment, while MRP 1, MRP 3, MRP 4 and MRP 5 are expressed on the basolateral compartment (Estudante et al., 2013; Hoosain et al., 2015).). P-gp is a transporter responsible for efflux of substrates back into the lumen. Furthermore, intestinal P-gp may contribute to the systemic clearance of intravenously administrated drugs by active secretion into the intestinal lumen (Sodani et al., 2012). P-gp is highly expressed on apical compartment of ileum and colon and less expressed into duodenum and jejunum (Sodani et al., 2012). Two major mechanisms have been described regarding P-gp drug–drug interactions are inhibition and induction (Estudante et al., 2013). MRP1 transporter transports drugs from the cell into the interstitial fluid which acts as an absorptive transporter and protects enterocytes from the accumulation of toxic chemicals (Estudante et al., 2013). It can transport hydrophobic drugs or other compounds that are conjugated or complexed to the anionic tripeptide glutathione (GSH), to glucuronic acid, or to sulfate (Estudante et al., 2013). MRP2 is localized on the apical side of enterocytes and transports leukotrienes and various glutathione conjugates, as well as conjugates of heavy metals including arsenic and cadmium (Estudante et al., 2013). Additionally, MRP2 also transports glucuronide and sulfate conjugates of several bile salts, a range of unconjugated organic anions (Sodani et al., 2012). BCRP is demonstrated to resist intestinal absorption of anti-cancer agents, sulfate conjugates, non-chemotherapy drugs. BCRP can also transport hydrophilic conjugated organic anions like the

sulfate conjugates, whereas P-gp generally transports hydrophobic compound (Estudante et al., 2013).

The SLC superfamily includes many pharmacokinetically important transporters at the intestine such as proton dependent oligopeptide transporters, organic anion transporting polypeptides (OATP), organic cation transporters (OCT), plasma membrane monoamine transporter (PMAT) and the monocarboxylate transporters (MCT) (Estudante et al., 2013). Peptide transporters expressed on the apical membrane of small intestine is PEPT1 (Lü & Klaassen, 2006). At least one peptide bond and a free terminal carboxyl group is a minimum requirement to be transported by PEPT1. OATPs are responsible for the transport of a wide range of organic molecules, including drugs, hormones, and various endogenous compounds, across cell membranes. In the intestine, OATPs play an important role in the absorption of certain drugs and other organic molecules from the gut lumen into enterocytes (intestinal cells) and, subsequently, into the bloodstream. The intestinal expression of OATP members is limited, with only one report suggesting the presence of OAT2 mRNA in mouse fetal intestine. Therefore, the role of the OATP family in the intestinal absorption of drugs is considered negligible (Estudante et al., 2013). OCT1 and OCT2 are expressed on the basolateral compartment of enterocytes in the small intestine, and they are responsible for uptaking and transporting of organic cations in the cytoplasm. They have a broad substrate specificity and can transport a wide range of organic cations, including drugs, endogenous compounds, and toxins. OCTNs facilitate the transport of organic cations across biological membranes. These transporters are essential for the body's absorption and distribution of a variety of endogenous and exogenous organic cations, including ions like dopamine and serotonin as well as some drugs and xenobiotics (Hoosain et al., 2015).

1.2 Enniatins

Enniatins (ENNs) are emerging mycotoxins that are produced by the filamentous fungi of *Fusarium species* (Ivanova et al., 2019) and these toxins can be found in a wide variety of food such as cereals, and their derivates, dried fruit, spices, cocoa, and coffee (Cimbalo et al., 2021). ENNs are chemically N-methylated cyclic hexadepsipeptides (Bhateria et al., 2022). Their toxicity is based on its ionophoric properties that they can integrate themselves in the biological membranes forming cation selective pores. They can transport monovalent and divalent cations, either in sandwiched complexes or by creating channels in biological membranes resulting the distrubance in the physiological balance (Pérez-Fuentes et al., 2022; Rodríguez-Carrasco et al., 2020). In a polar environment, the amide carbonyls point away from the plane of the molecule, and on the other side, the ester carbonyls and N-methyl groups point in the opposite direction. In a non-polar environment, all of the polar residues point to the center of the molecule. This three-dimensional structure of ENNs is what gives them their hydrophilic and hydrophobic properties. These hydrophilic and hydrophobic properties of ENNs enable their extraction using different solvents with polarities ranging from methanol to chloroform. ENNs are protected from the nonpolar solvent, as the apolar phenyl-, isopropyl-, secbutyl-, and methyl-groups point to the outer surface of the molecule where they can form hydrophobic interactions with membranes or organic solvents. Due to the apolar side chains directing out from the outer surface of the molecules, ENNs are considered as lipophilic compounds (Bertero et al., 2020).

There are 29 ENNs analogs identified. The frequently found ENNs in food and feed are ENN A, ENN A1, ENN B, and ENN B1, Figure 3 (Bertero et al., 2020; Pérez-Fuentes et al., 2022). While enniatin A has three sec-butyl substituents, enniatin A1 has two sec-butyl and one iso-propyl group. Similarly, enniatin B has three iso-propyl substituents, whereas enniatin B1 has two iso-propyl groups and one sec-butyl group (Svingen et al., 2016). The molecular weight of ENN A, ENN A 1, ENN B, ENN B1 are 681.9, 667.9, 639.8, and 653.8 g/mol respectively (PubChem, 2023).

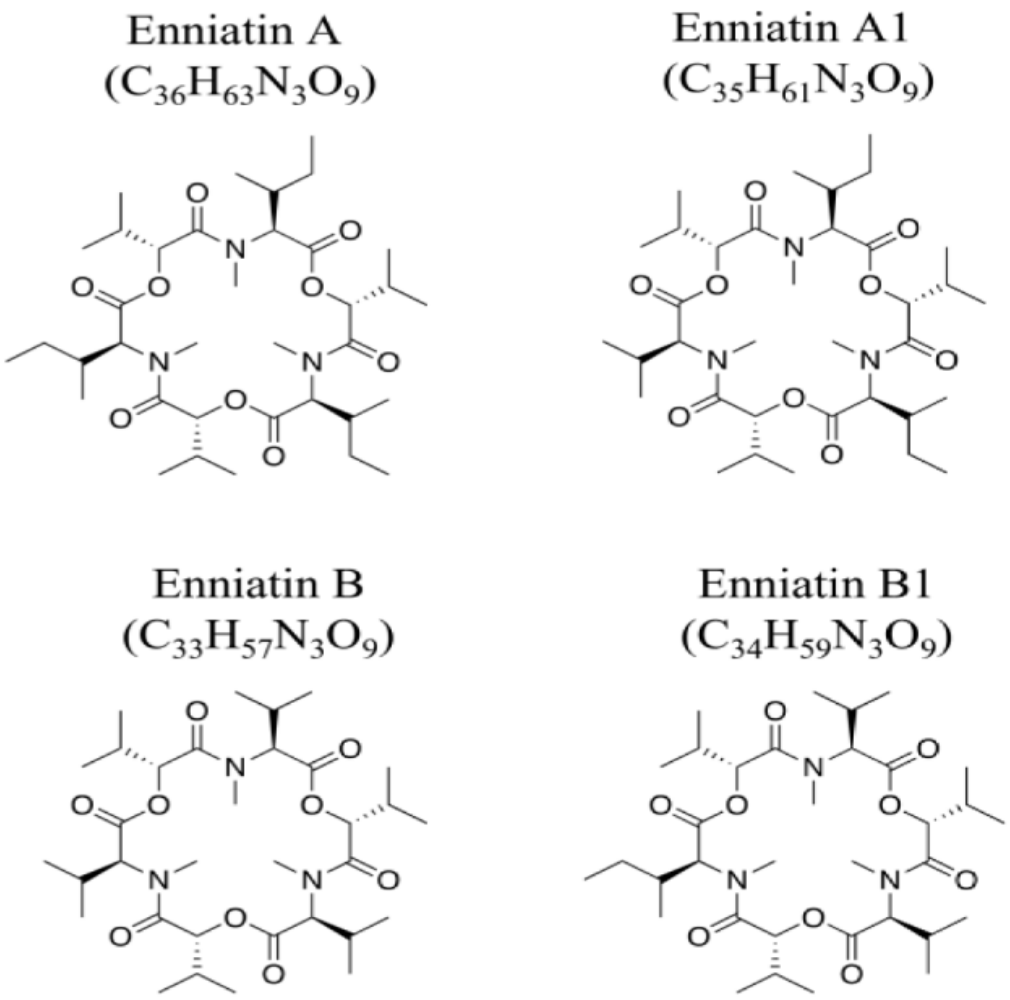

Figure 3. Enniatin A, A1, B, and B1 structure (De Felice et al., 2023).

1.2.1 Kinetics of ENNs

ENNs are ingested through contaminated food in the body. Ingested ENNs enter the stomach where they undergo various biological processes and interaction. These processes isolate ENNs from the food. Isolated ENNs go through the absorption in the small intestine after passing stomach Figure 4. Absorption of ENNs through epithelium can occur through passive diffusion or active transport mechanism. N-methylation of the peptide backbone of ENNS can be an effective approach to increasing the passive diffusion of cyclic peptides across membranes

(Dougherty et al., 2019). The ionophore properties of ENNs defines their absorption in epithelium through transcellular pathway, a passive diffusion (Picci et al., 2022). Besides passive diffusion, active transporter such as OATPs, a SLC transporters could play a role for uptake of ENNs into cytoplasm of enterocytes from lumen (Dougherty et al., 2019). In enterocytes, ENNs may be involved in phase I metabolism by mainly metabolized by CYP3A4 enzyme (see appendix 1 for intestinal enzymes).

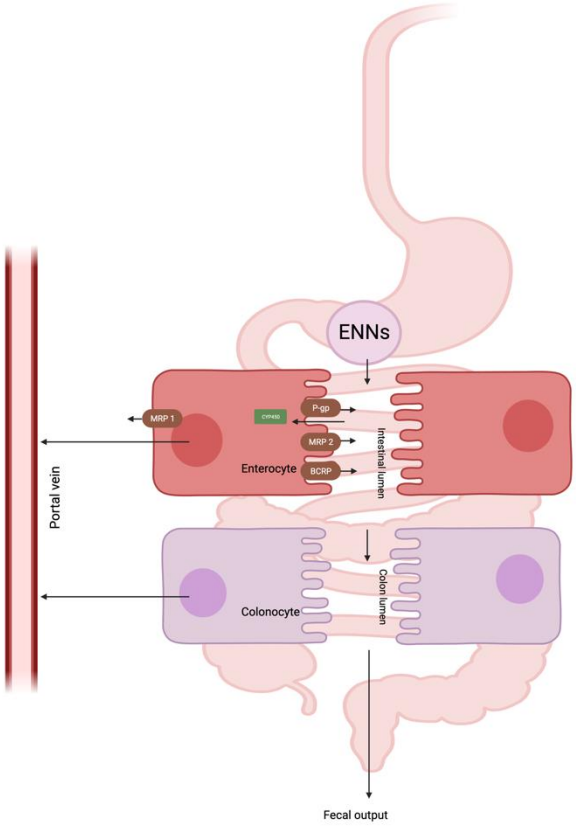

Figure 4.Absorption, digestion, metabolism, and excretion of enniatin in human body.

CYP3A4 was reported as an important enzyme for ENN B metabolism and the metabolites are the products of the oxidative reaction such as Mono-oxygenated, di oxygenated, and demethylated. ENN B metabolites were reported as more hydrophilic than their parent compound (Fæste et al., 2011). Similar subtype CYP3A4 was also reported by Bhateria et al. (2022) for the mainly metabolism of ENN A followed by CYP1A2. Ivanova et al. (2019) also reported subtype CYP3A4 involved into most ENN B1 metabolism. Similarly, Rodríguez-Carrasco et al., (2018) also reported the main enzyme involve in ENN B1 metabolism as CYP3A4,

followed by CYP1A2 and CYP2C19. ENN B1 metabolites are products of hydroxylation, carbonylation, carboxylation, and oxidative methylation reactions that are the product of oxidative reaction (Ivanova et al., 2019). However, metabolism of ENN A1 has not been discovered to this date. Above studies on ENNs metabolism pathway show that ENNs go through mainly oxidation in phase I metabolism, prominently metabolized by the subtype CYP3A4 enzyme.

Ivanova et al., (2010) reported P-gp and MRP2 as the substrates of ENN B1. P-gp and MRP2 transport their substrate from enterocyte to the lumen. Therefore, ENNs metabolites may be effluxed from enterocytes (cell) to lumen by the ABC transporters such as P-gp, MRP 2, and BCRP (Ivanova et al., 2010; Bertero et al., 2020). Once ENNs metabolites are effluxed to the lumen, they pass through the large colon. Some enniatin metabolites may not be absorbed efficiently in the intestine and can be excreted in feces. This is more likely for metabolites that are large, highly polar, or poorly absorbed (Ojiro et al., 2023). The transport of ENNs in small intestine is quite complex and only few studies have been done in vivo and in vitro as shown in, Table 1. *In vivo* oral bioavailability[2] studies show that ENNs are quicky absorbed, distributed, and excreted compared to *in vitro* studies done in mammals. However, *in vivo* studies on ENNs transport in small intestine in rodent is limited. Rodent are used as similar model to human physiology for the toxicology studies (Bhateria et al., 2022).

[2] 'bioavailability' refers to the 'availability for absorption or bioaccessibility', 'absorption', 'tissue distribution' and 'bioactivity' (Meca et al., 2012).

Table 1 Literature study of ENNs oral bioavailability in vivo and in vitro models.

Literature	Type of study	Finding	Reference
Pharmacokinetics and 28-day repeated-dose toxicity of enniatin B after oral administration in mice	In vivo study	In the oral dose group of Five-week-old male mice, an average of 5.26% of the amount of ENNB administered was detected in the feces between 4 h and 24 h post-dose when a single oral dose of ENNB at 30 mg/kg BW was admistrated. high fecal ENNB content supports the kinetic analysis of ENNB estimated for its high oral bioavailability (139.9%) in mice.	(Ojiro et al., 2023)
Pilot toxicokinetic study and absolute oral bioavailability of the *Fusarium* mycotoxin enniatin B1 in pigs	In vivo study	The experimental piglets were orally administrated ENNB1. Blood sample showed rapid absorption after oral intake with oral bioavailability of 90.0%. Furthermore, ENN B1 was rapidly distributed and eliminated as well.	(Devreese et al., 2014)
Enniatin B_1 is a substrate of intestinal P-glycoprotein, multidrug resistance-associated protein 2 and breast cancer resistance protein	In vitro study: Caco-2 cell, Madin-Darby canine kidney cell line (MDCK II)	The study showed ABC transporters such as P-gp, MRP2 and BCRP involvement in efflux of ENN B1in small intestine. Uptake of ENN-B1 was reported 6.7 times higher in the Basolateral (BL)→ apical (AP) direction as compared to the opposite direction, indicating involvement of apically located transporters. The inhibitors of Pgp (Verapamil) and MRP2 (MK571) significantly increased the transport of ENN-B1 in the AP→BL direction, while inhibition of BCRP (FTC)) only weakly and not significantly increased ENN-B1 transport in this direction. This suggested that at least two ABC transporters were involved in ENN-B1 transport. Furthermore, ENN-B1 was reported to act as an inhibitor of both Pgp and MRP2 in over expressing MDCK II cell line. However, ENN-B1 acted as BCRP substrate, but only in a low BCRP inhibitory potential.	(Ivanova et al., 2010)
Study of the potential toxicity of enniatins A, A1, B, B1 by evaluation of duodenal and colonic bioavailability applying an in vitro method by Caco-2 cells	In vitro study: Caco-2 cell	Bioavailability of the ENs A, A1, B and B1 was expressed as absorption value in duodenal after 4 h of exposure ,76.8 for EN A, 70.2 % for EN A1, 67.0 % for EN B, and 62.2% for EN B1 when 1.5 µM concentrations were added. after 4 h of exposure, 57.7 for EN A, 68.8 % for EN A1, 65.0 % for EN B, and 65.1% for EN B1 when 3 µM concentrations were added. Higher concentration injection decreased the transport of ENN A, ENN A1, and ENN B except for ENN B1. However, transport of ENNs increased with the time for both injected concentrations. The least transported to apical was reported to be ENN A. The study showed that less ENNs were absorbed in small intestine, whereas large number of ENNS were absorbed in large intestine.	Meca et al., (2012)

1.2.2. Scientific and societal relevance

The presence of mycotoxins in feed and food has increased over time due to climate change (Van Der Fels-Klerx et al., 2012; Novak et al., 2019). ENNs are known as antibacterial, anthelmintics, antifungal, herbicidal, insecticidal, and immunotoxic (Bertero et al., 2020; Bhateria et al., 2022). Moreover, ENNs have shown several cytotoxic effects at very low micromolar range in different cell lines (Pérez-Fuentes et al., 2022; Prosperini et al., 2017). However, it has been reported in vivo studies that ENNs have no or low toxicity (Ojiro et al., 2023); Rodríguez-Carrasco et al., 2018). According to European Food Safety Authority (EFSA) scientific opinion, no specific reports on human toxicosis caused by ENNs have been identified in Europe or elsewhere in the world (EFSA, 2014). Furthermore, there have been no TDI, or acute reference dose (ARfD) drawn by EFSA due to insufficient data of ENNs toxicity. However, EFSA has established threshold of toxicological concern (TTC) approached according to Cramer class III for the sum of ENNs as 1.5 µg/kg bw per day. Moreover, mixture of ENNs has higher cytotoxicity in Caco-2 cell than individual ENNs analog (EFSA), 2014).

The European Food Safety Authority (EFSA) has emphasized the importance of improving our understanding of the oral bioavailability of ENNs. This is because the absorption of ENNs in the intestines is likely a key factor in their *in vivo* toxicity. The degree of absorption of ENNs through the gastrointestinal tract varies depending on the specific type of ENN. However, lack of knowledge about the varying permeabilities of different ENNs, as the mechanism by which ENNs are transported across intestinal tissue from the gut lumen to the portal vein is not fully understood. Meca et al., (2012) studied the transport of ENNA, ENNA1, ENNB, and ENNB1 in Caco-2 cell monolayer, however, there has not been any study done till this date regarding apparent permeability (P_{app}).

Caco-2 cell monolayer is derived from human colon adenocarcinoma cell line, most widely used in *in vitro* intestinal models for predicting human intestinal drug absorption (Ivanova et al., (2010); Meca et al., (2012)). P_{app} is the rate at which toxic chemical or drug, can travel through biological membranes barrier such as gastrointestinal tract, skin, or respiratory system. P_{app} is used to determine how much substance can traverse these barriers and enter the bloodstream or other tissues. These outputs are used to predict the kinetics of the chemical in animal or human body, and eventually the output is used in the risk assessment.

This project aimed to study ENNs transportation in terms of permeability in differentiated human intestinal model for input into physiologically based (PBK) models of ENNs to predict the variability in blood and tissue concentrations of ENNs in humans for toxicological risk assessment.

2. Materials and methods

2.1. Chemicals

MEM (1X) (Gibco, Thermo-Fisher cat.no.21090022), FBS (Foetal Bovine Serum) Heat inactivated; Different sources 20% (2 tubes of 50ml), PSG (Penicilline/glutamine solution); 1% (5.5ml), PY (Pyruvate) (aliquoted in -20°C); 1% (5.5ml), Dimethylsulfoxide (DMSO) [Merck, Germany,102952, ACS grade], Acetonitrile [ACN- 001204102BS, Formic acid [(99-100%) - VWR chemicals/ 20318.297P], Hank's balanced salt solution (HBSS) without phenol red [Gibco/14025-050], HEPES [Sigma Aldrich, A2153-100G], Rhodamine 123 powder : molecular weight=380.82 Da (Cas no. 62669-70-9) (AFSG.BZ.124.TOX.4098.VCC2), Fluorescein powder : molecular weight 332.31 Da Lot 052K3470 EC no 2190318, 10mM ENNA, 10 mM ENNA1, 10 mM ENNB, 10 mM ENNB1.

2.2. Cell culture of Caco-2

Human colon carcinoma (Caco-2) cells were cultured in culture medium (MEM, FBS, PSG, PY) in 50ml flask. Cells at confluence of 80% were washed with pre-warmed PBS to remove the dead cells. Cells were trypsinized afterwards to detach the cells. The detached cells were diluted with culture medium and incubated at 37°C in an atmosphere of 5% Co_2.The passage of caco-2 cells was done twice a week. The passage numbers of 8-20 were used for the transport study (see appendix 3 for detailed protocol).

2.3. Caco-2 seeding in transwell.

Caco-2 cells seeding in 12-transwells polystyrene plates (Sigma; thincerts Greiner; transwells Corning, 12 mm Transwell with 0.4 µm pore with an area of 1.12 cm^2) and 24 transwell (Sigma; thincerts Greiner; transwells Corning, 6.5 mm Transwell with 0.4 µm pore polycarbonate membrane insert with an area of 0.33 cm^2) were done once the confluence was >80%. Before seeding, cells were washed with PBS and trypsinized for 5-10 minutes to detach from the flasks. Trypsinized cells were measured in cello meter to calculate the total live cell count. Cell suspension of $4.48*10^5$ cells/ml (200000 cell/cm^2) and $3.3*10^5$ cells/ml (100000 cell/cm^2) were prepared in culture medium for 12-transwell and 24 transwell respectively. The working volume of 0.5ml and 1.5ml were used for apical and basolateral compartment respectively for 12-well where 0.5ml of cell

suspension was added to apical (insert) and 1.5 ml of transport medium was used in basolateral. Similarly, the working volume of 100ul and 600ul were used for apical and basolateral compartment respectively for 24-transwell where 0.1ml of cell suspension was added to apical (insert) and 600ul of culture medium was used to basolateral. The seeded transwells were incubated at 37°C in an atmosphere of 5% Co_2 for 21 days to differentiate caco-2 monolayer. Culture medium of seeded transwell was changed three times in a week with pre-warmed culture medium (see appendix 4 for detailed protocol).

2.4. Investigation of plastic binding of Enniatins and rhodamine 123

Plastic binding test was done in 12-transwell without cell to investigate the test compound ENNs and rhodamine 123 binding to plastic. Freshly prepared 0.5ml exposure of 1uM ENNs mixture in transport medium (0.25uM ENNA, 0.25uM ENNA1, 0.25uM ENNB, 0.25uM ENNB1) was loaded to apical compartment and 1.5ml of 1uM ENNs mixture in DMSO was loaded to basolateral compartment. Similarly, 0.5ml of 5uM rhodamine in transport medium was loaded to apical compartment and 1.5ml transport medium was loaded to basolateral compartment. Rhodamine 123 and ENNs were exposed in 2 and 3 technical replicates respectively. The exposed transwell was incubated at room temperature for 4 hours. After the exposure time, 300ul samples were collected from apical and 300ul samples from basolateral compartment. ENNs samples were stored at -20 °C for LCMS analysis while rhodamine 123 was measured on the same day using fluorescent (Tecan) (See appendix 5 for detailed protocol).

2.5. Transport assay

Transport assay was done on the 21 days of post seeding of Caco-2 cell in transwell Figure 5.

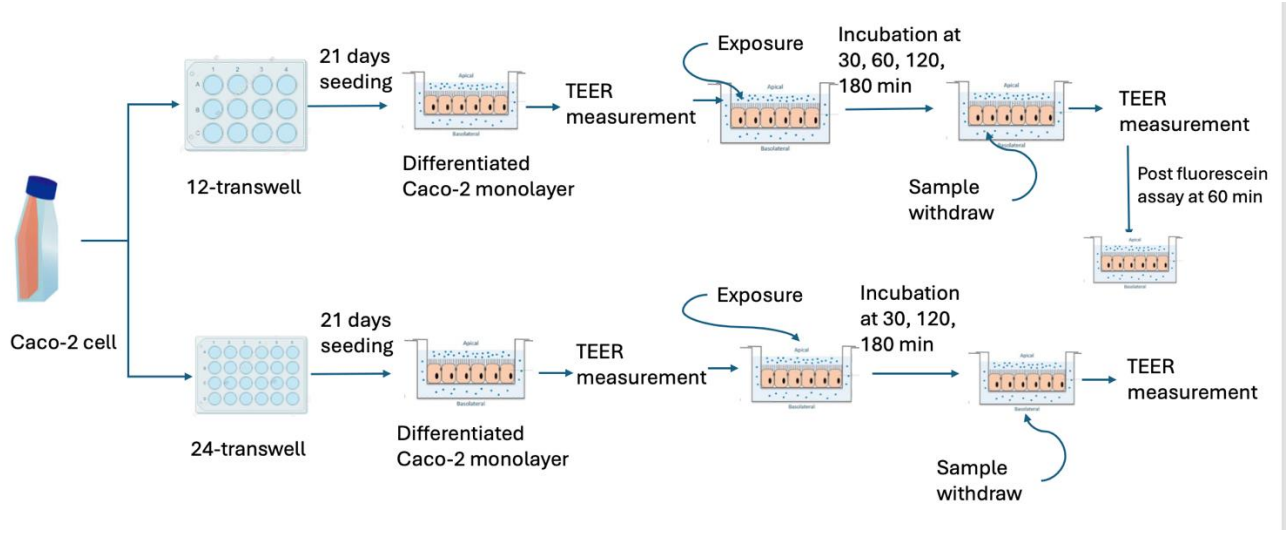

Figure 5. Permeability assay in differentiated Caco-2 seeded transwells.

The transport assay was upgraded according to the protocol done by Hubatsch et al. (2007). Culture medium from transwell was aspirated from basolateral and apical compartment. Pre-warmed transport medium was loaded to apical and basolateral as per transwell's working volume and incubated at 37°C in an atmosphere of 5% Co_2 for 30 minutes. Tissue transepithelial electrical resistance (TEER) was measured in 30 min of incubation time. After 30 minutes, transport medium was aspirated from basolateral and apical compartment. 1uM ENNA in DMSO, 1uM ENNA1 in DMSO, 1uM ENNB in DMSO, and 1uM ENNB1 in DMSO were used for exposure solutions. 5uM, 10uM or 50uM rhodamine 123 was used as positive control to investigate the functional expression of P-gp in Caco-2 cell monolayer cultured in transwell. 5uM fluorescein was used only in one experiment conducted in 12-transwell as fluorescein post-control assay to assess cell integrity because it exhibits extremely low permeability (Ahmed et al., 2020). DMSO was used as negative control to investigate solvent intervene in transport of compounds in Caco-2 cell monolayer. All the exposure solutions were exposed in apical compartment of transwell in two technical replicates. The basolateral compartment was loaded with transport medium to balance the apical volume. The exposed transwell were incubated for 30 minutes, 60 minutes, 120 minutes, and 180 minutes at 37°C in an atmosphere of 5% Co_2. At each time point, samples were collected from the basolateral compartment and replaced with transport medium with same volume the sample was collected. At the final time point, samples were taken from apical and basolateral compartment. ENNs samples were stored at -20 °C for LCMS analysis while rhodamine 123 was measured on the same day using fluorescent (Tecan) (See appendix 7, 8 for permeability assay in 12-transwell with cell and 24-transwell with cell).

The concentration measured for the compounds were used to determine recovery, cumulative permeated amount and apparent permeability (P_{app}).

P_{app} for the compound was calculated using the equation 1.

$$\mathrm{Papp} = \frac{\mathrm{Q}}{\mathrm{A\ C\ C\ C\ t}} \qquad \text{equation 1}$$

Where Q is the cumulative amount of compound permeated in (nmol), A is surface area of insert in (cm^2), C is initial amount of compound in apical compartment at time 0 (uM), and t is time of the experiment (s).

2.6. Caco-2 monolayer integrity assessment

The integrity of Caco-2 cell monolayer was assessed by TEER measurement using the Millicell® ERS-2 Epithelial Volt-Ohm Meter (Millipore, Amsterdam, The Netherlands). TEER measurements were performed before the exposure of the compounds and after the transport assay. TEER was indicated as Ω x cm^2. TEER of >300 Ω x cm^2 were used for the transport assays as they were considered to have a tight cell barrier (Senarathna & Crowe, 2015; Troutman & Thakker, 2003; Chen et al., 2015).

2.7. Fluorescein post control assay

Fluorescein post control assay was done using 5uM fluorescein in transport medium to investigate cell integrity during the transport assay. This was done after the transport assay in 12-transwell. The apical and basolateral compartment were aspirated and washed with transport medium with the working volume of 0.5 to apical and 1.5ml to basolateral compartment. The transport medium was aspirated, and apical compartment of all transwell were loaded with 0.5ml 5uM fluorescein in DMSO. 1.5ml transport medium was loaded to basolateral and the exposed transwell was incubated at 37°C in an atmosphere of 5% Co_2 for 60 minutes. 100ul sample was drawn from apical and 100ul sample from basolateral compartment. The collected samples were measured in Tecan (see appendix 7 for detailed protocol).

Rhodamine 123 and fluorescein samples were measured in 96 transwell {Greiner 96 Flat Bottom Black Polystyrene Cat. No.: 655090/655096/655097 [GRE96fb_µClear.pdfx] } together with freshly prepared rhodamine 123 and fluorescein standard solution for calibration respectively (appendix 6). The measurement was done in Tecan with excitation of 505nm and emmision of 525nm for rhodamine 123 and with excitation of 437nm and emmision of 515nm for fluorescein.

Post Fluorescein transport was calculated in percentage using equation 2.

$$\text{transport } \% = \frac{\text{Total amount tranported}}{\text{Initail amount}} \text{ X100} \qquad \text{equation 2}$$

Where total amount transported is the amount of fluorescein measured on basolateral compartment in nmol. Initial amount is the amount of fluorescein exposed on apical compartment in nmol.

2.8. Profiling of ENNs by LC/MS/MS

ENNs samples were analyzed in Shimadzu LCMS 8045 (1 and 2) (LC Column**:** Kinetex C18 1.7 µm x 100 Å x 150 mm x 2.1 mm (Phenomenex), Guard Column Holder (Phenomenex AJ0-9000), Guard Column (Phenomenex AJ0-8782)) with flow rate of 0.3ml/min. MilliQ Water with 0.1% Formic Acid and Acetonitrile with 0.1% Formic Acid were used as mobile phase.

Recovery was calculated using equation 3 for transport assay done with cells.

$$\text{Recovery } \% = \frac{\text{Total amount recovered}}{\text{Initail amount}} \text{ X100} \qquad \text{equation 3}$$

Where total amount recovered is the amount of compound measured on the apical and basolateral compartment in nmol. Initial amount is the amount of compound exposed on apical compartment in nmol.

2.9. Statistical analysis

The experiments were conducted in two-three technical replicates (n=2, n=3) and data were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). Data were analyzed in GraphPad Prism version 10.1.1 (270).

3. Results

3.1. Plastic binding of Enniatins and rhodamine 123.

Plastic binding was assessed for rhodamine 123 in 12-transwell and ENNA, ENNA1, ENNB, and ENNB in 12-transwell and 24-transwell, calculated based on recoveries of the nominal concentration. The result shows that rhodamine 123 was recovered 81.94% ± 8.44 at 4 hours in the well of 12-wells Transwell plate without cell Figure 6.

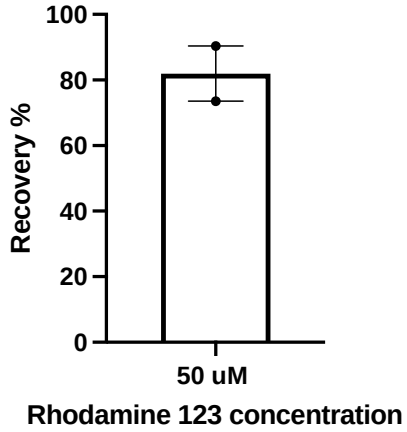

Figure 6. Recovery of 50uM Rhodamine 123 in 12 transwell without cells at 3 hours of transport. n=2, ± SEM

ENN A1 showed highest recovery with 65.1% ± 15.2 in 12-transwell followed by ENNB and ENNA with 43.2%± 10.67 and 17.09 ± 9.78 respectively. While ENNB1 showed lowest recovery with 14.6%± 2.96 at 4 hours Figure 7A. Similar results were observed when ENNs the ENNs recoveries were assessed and calculate from 24-transwell at 2 hours in the transport medium. The lowest recovery was shown for ENNA with 52.46% ± 16.16 and highest recovery for ENNB with 92.64% ± 87.84. Next to that, ACN used to extract the empty transwell after the experiment increased ENNs total recoveries leading the highest recovery for ENNB with 119.34% ± 13.8 and the lowest recovery for ENNA1 with 75.18%. ± 0.58 (Figure 7B). The highest extracted ENNs from the transwell was ENNA (36%) while the lowest extracted ENNs was ENNA1 (14%).

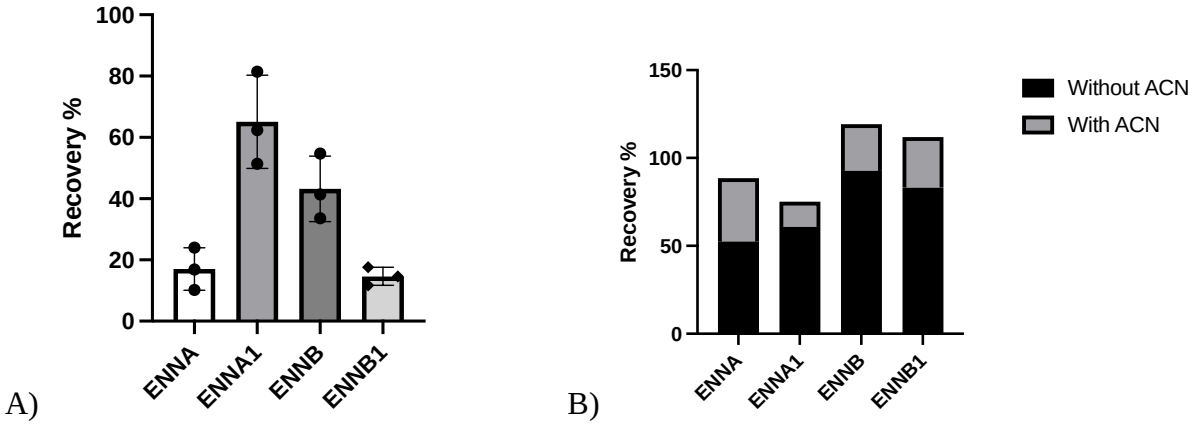

Figure 7. A) 0.25 uM ENNA, 0.25uM ENNA1, 0.25uM ENNB, and 0.25uM ENNB1 recovery in 12-well at room temperature at 4 hours. n=3± SD, B) 1uM ENNA, 1uM ENNA1, 1uM ENNB, and 1uM ENNB1 recovery with ACN and without ACN in 24-transwell without cells at 4 hours at 37C, 5% Co2. , n=2±SEM.

3.2. Transport assay

3.2.1. Cumulative permeated amount of rhodamine 123 and Enniatins at 3 hours of transepithelial transport

The cumulative permeated amount of 5uM and 50uM rhodamine 123 increased with time in both 12-transwell and 24-transwell respectively from Apical to basolateral (A->B) (Figure 8A, B). The cumulative permeated amount of 5uM rhodamine 123 was 6 times higher in 12-transwell than 50uM rhodamine 123 in 24-transwell.

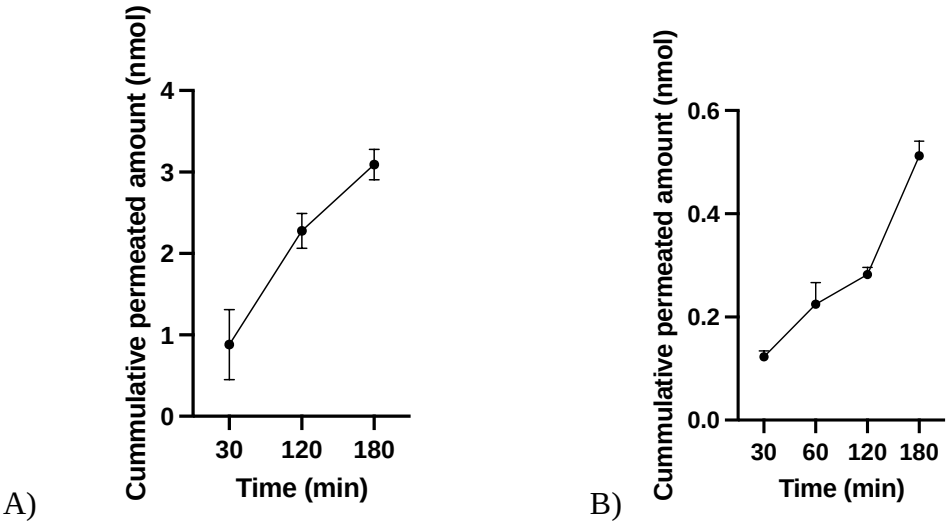

Figure 8. A) Cumulative permeated amount of 5uM rhodamine 123 in 12-transwell with cell at 30, 120 and 180 minutes from A->B. n=2 ± SEM. B) Cumulative permeated amount of 50uM rhodamine 123 in 24-transwell with cell at 30, 60, 120 and 180 minutes from A->B. n=2± SEM.

ENNs also showed consistent trend in transepithelial transport in 12-transwell at different time points, increased with time (Figure 9 A). At 3 hours of transepithelial transport ENNB showed the highest transportation from A->B with 0.07 nmol ± 0.01 while ENNA1 showed the lowest transepithelial transport with 0.005 nmol ± 0.0001. However, ENNs transepithelial transport in 24-transwell did not show consistent trend but the cumulative permeated amount increased with time (Figure 9 B). Like in 12-transwell, ENNA1 was the lowest transported at 3 hours of transepithelial transport with 0.002 nmol ± 0.0007 while ENNA was the highest transported with 0.004 nmol ± 0.0002 (only one ENNB sample at 30 min time point was detected in LCMS). The cumulative permeated amount of all ENNs was almost 10 times higher in 12-transwell than in 24-transwell.

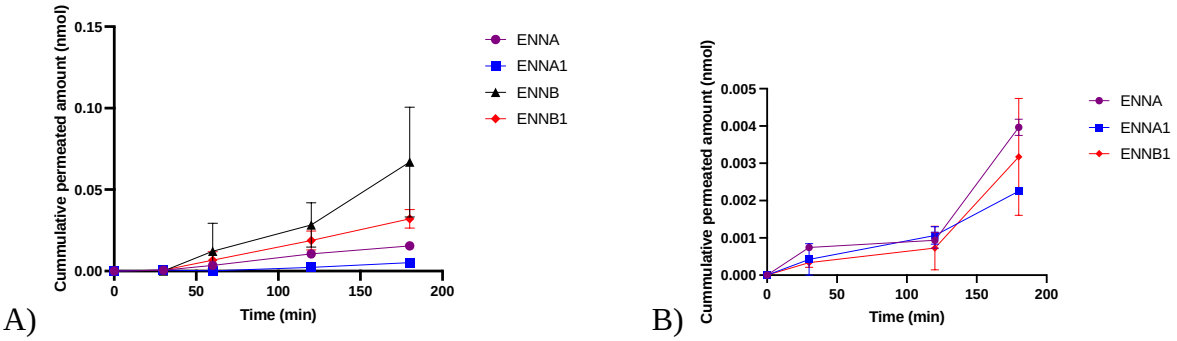

Figure 9. Cumulative permeated amount of 1uM ENNA, 1uM ENNA1, 1uM ENNB, and 1uM ENNB1 in 12-transwell with cell at 30, 60, 120 and 180 minutes. n=2 ± SEM. B) Cumulative permeated amount of 1uM ENNA, 1uM ENNA1, 1uM ENNB, and 1uM ENNB1 in 24-transwell with cell at 30, 120 and 180 min.

3.2.2. Apparent permeability of Rhodamine 123 and Enniatins

Apparent permeability from A->B decreased with the lower concentration of rhodamine 123 used in transport assay at 30 min in 24-transwell (Figure 10). 50uM rhodamine, 10uM, and 5uM rhodamine 123 P_{app} are 0.41± 0.04 *10^{-5} cm/s, 9.31± 2.06 10^{-5} cm/s, and 11.8 ± 1.49 10^{-5} cm/s respectively. On the other hand, 5uM rhodamine 123 had P_{app} of 8.76 ± 4.28 * 10^{-5} cm/s in 12-transwell at 30 min. 5uM rhodamine 123 P_{app} was three times higher in 24-transwell compared to 12-transwell.

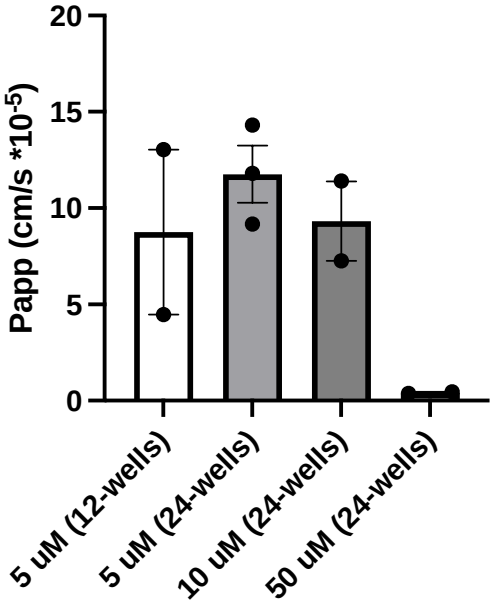

Figure 10. Apparent permeability of rhodamine 123 in transwell with cell at 30 min. n=2 ± SEM.

In 12-transwell, ENNB showed the highest P_{app} of 5.53 ± 0.98 * 10^{-6} cm/s followed by ENNB1, and ENNA with 2.65 ± 0.17 and 1.28 ± 0.02 respectively while ENNA1 had lowest P_{app} of 0.43 ± 0.01 * 10^{-6} cm/s (Figure 11 A). However, ENNA had the highest P_{app} of 1.11± 0.03 * 10^{-6} cm/s followed by ENNB1 with 0.89 ± 0.002 * 10^{-6} cm/s while ENNA1 had the lowest P_{app} of 0.63±0.01 * 10^{-6} cm/s (Figure 11 B). ENNB (n=1) at 30 min had P_{app} of 0.6 * 10^{-6} cm/s. The results shows that ENNA and ENNA1 in 12-transwell and 24-transwell have the around same P_{app} values.

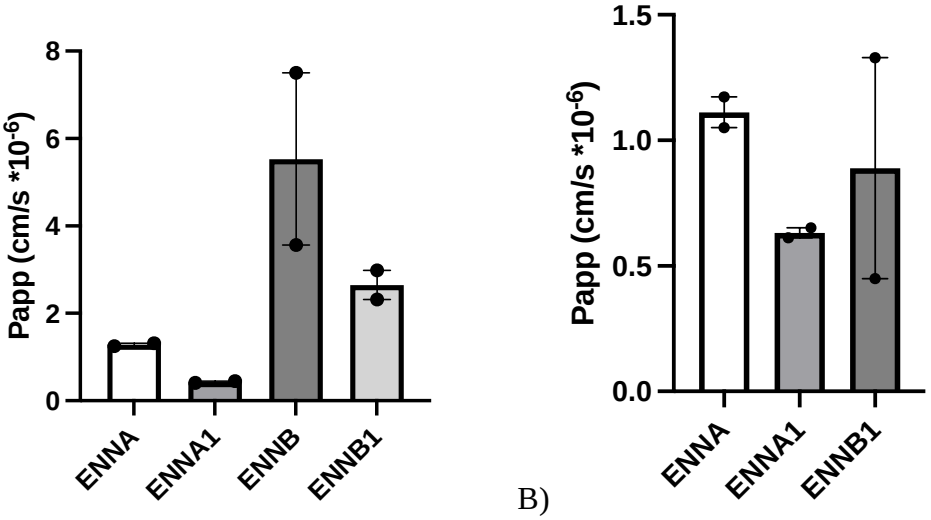

Figure 11. Apparent permeability of 1uM ENNA, 1uM ENNA1, 1uM ENNB, and 1uM ENNB1 in 12-transwell with cell at 180 min n=2 ± SEM. B) Apparent permeability of 1uM ENNA, 1uM ENNA1, 1uM ENNB, and 1uM ENNB1 in 24-transwell with cell at 180 min. n=2 ± SEM.

3.3. Caco-2 monolayer integrity during transepithelial transport assay

3.3.1. Caco-2 monolayer integrity assessment using TEER.

The TEER of Caco-2 cell monolayer in 12-transwell after the transport assay were >139% in the transwell where compounds were exposed (Figure 12 A). However, in 24-transwell only rhodamine 123 exposed cells exceeded 100% TEER with 108.86% ± 12.98 after the transport study while ENNB had the lowest TEER% with 47.77%±3.53 followed by ENNA, with 68.77±12.45 (Figure 12 B). DMSO, ENNB1 and ENNB had >85% TEER with 88.19±8.78, 90.34±11.83, and 94.32±11.08 respectively. TEER % in 24-transwell where 5uM ,10uM rhodamine 123 including medium control (only cells) were exposed for 30 min transport assay had <100% TEER (Figure 12 C). The TEER% for 5uM rhodamine 123, 10uM rhodamine and Blank were 55.94 ± 31.04, 57.96±35.8, and 80.16± 23.8 respectively.

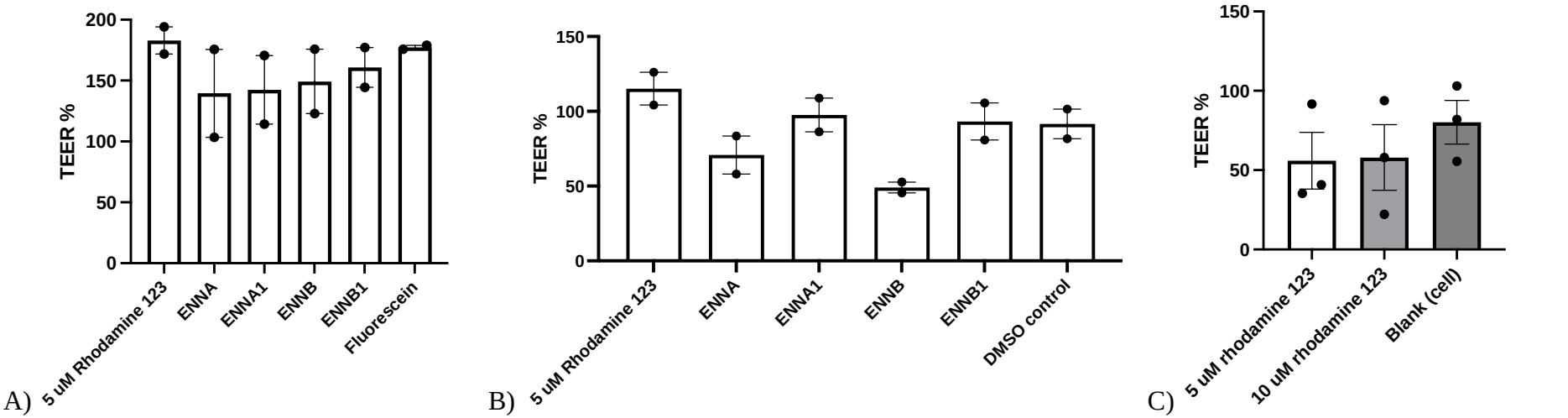

Figure 12. A) TEER% of Caco-2 seeded transwell in 12-transwell after transport assay of 5uM rhodamine 123, 1uM ENNA, 1uM ENNA1, 1uM ENNB, 1uM ENNB1 and 1uM fluorescein at 180 min, n=2 ± SEM, B) TEER% of Caco-2 seeded transwell in 24-transwell after transport assay of 5uM rhodamine 123, 1uM ENNA, 1uM ENNA1, 1uM ENNB, 1uM ENNB1 and DMSO at 180 min, n=2 ± SEM. C) TEER% of Caco-2 seeded transwell in 24-transwell after transport assay of 5uM rhodamine 123, 10uM rhodamine 123 and DMSO at 30 min, n=2 ± SEM

3.3.2. Fluorescein post control assay

Post fluorescein transport in 12-transwell where compounds were exposed and transepithelial transported for 3 hours showed <1% fluorescein transport in 5 uM rhodamine 123, fluorescein, ENNA and ENNB exposed transwell (Figure 13). However, ENNA1, and ENNB1 exposed transwell showed >1% fluorescein transport with 1,05%±0,42 and 2.52%±1.74 respectively.

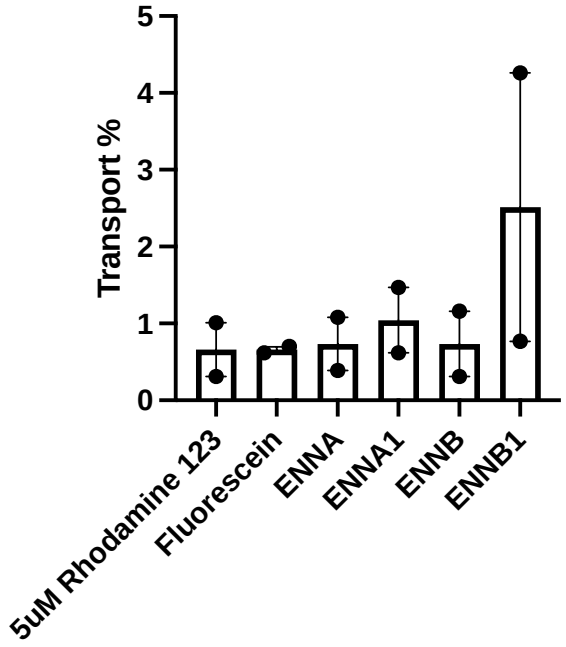

Figure 13. Fluorescein post transport assay in 12-transwell after transport study of 5uM rhodamine, fluorescein, 1uM ENNA, 1uM ENNA1, 1uM ENNB, and 1uM ENNB1, n=2 ± SEM.

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4. Conclusion and discussion

4.1. Plastic binding of Enniatins and rhodamine 123.

Rhodamine recovery of >80% in 12-transwell shows that Rhodamine doesn't bind to plastic (Bertero et al., 2020). On the other hand, ENNs recoveries of < 80% shows that these compounds bind to the plastic as experimented in 12-transwell. However, ENNA1, ENNB and ENNB1 >80% of recoveries in 24- transwell show that these compounds don't bind to plastic. Except for ENNA that has <70% recovery showed binding to plastic. However, ENNs extraction from same transwell (24-transwell) proves the binding of ENNs to plastic. The ENNs extraction in 24-wells also validates the binding of ENNs in 12-transwells where ENNA was recovered less compared to other ENNs in 12-transwell and was extracted highest in 24-transwell. The total ENNs concentration quantification in 24-transwell from recovery and extraction could be due to unreliable ENNs calibrations used for the sample analysis. The plastic binding results from both experiments suggest that ENNA has highest binding to the plastic while ENNA1 has lowest binding to the plastic. This could be explained by the fact that transwell surface is coated with hydrophobic polymer to support cell growth, and when ENNs is exposed to the transwell, butyl group in ENNs chemical structure might interact to the hydrophobic surface of transwell leading binding to the plastic (Proença et al., 2021; Svingen et al., 2016). The more butyl group in the chemical structure, the more a compound is lipophilic in nature and more interaction to hydrophobic surfaces (Bertero et al., 2020). According to lipophilicity, ENNA1 should show more plastic binding than ENNB and ENNB1. It could be possible that some ENNA1 attached to inside of plastic or went to the bottom, which couldn't be quantified. Only chemical adsorbed to the plastic surface is quantified.

4.2. Transport assay

4.2.1. Cumulative permeated amount of rhodamine 123 and Enniatins at 3 hours of transepithelial transport

The result on cumulative permeated amount for rhodamine 123 and ENNs shows that the permeated amount increases with time. The differences in cumulative permeated amount in 12-transwell and 24-transwell could be due to different plates used in the transport assays. In this study, the insert area of 12-transwell (1.12 cm^2) was almost four times larger than 24-transwell (0.33 cm^2). Larger insert area has more room for cell monolayer, thus may increase the transportation (Ghaffarian & Muro, 2013). Rhodamine 123 uptake across biological membranes is predominantly via paracellular passive route (Troutman & Thakker,2003; Forster et al., 2012). In larger insert area, more cell monolayer is formed, thus more tight junctions, resulting more transport of rhodamine 123 into

the basolateral compartment. However, in the transport studies, 5uM Rhodamine 123 exhibited similar transportation in both 12-transwell and 24-transwell showing no effects due to plates sizes as mentioned above (Troutman & Thakker,2003; Senarathna & Crowe, 2015). It could be that the different cell strain used in 12-transwell did not differentiate properly which could have increased rhodamine 123 epithelium transportation (Caro et al. 1995).

ENNs are lipophilic compounds, thus accumulate in the cells. According to ENNs lipophilicity, ENN A and A1 are found in higher concentrations in the cells and ENN B and B1 in the basolateral compartment (Bertero et al., 2020). The order of highest cumulative permeated amount can be seen in this study as well in 12-transwell for ENNB followed by ENNB1, ENNA and ENNA1. However, in 24-transwell, the permeated amount was higher for ENNB followed by ENNB1, ENNA and ENNA1. In a transport study done by Meca et al. (2012), the highest permeated concentration was found for ENNA followed by ENNA1, ENNB and ENNB1. The permeated concentration varied when the exposure concentration was increased from 1.5uM to 3uM, where the highest permeated concentration was found for ENNA1 followed by ENNB1, ENNB and ENNA. These results do not comply with the lipophilicity indication in transportation of ENNs as stated above literature. Therefore, not only the lipophilicity of the compounds but also the exposure concentration could have affected the permeation of the compounds during transportation.

4.2.2. Apparent permeability

Caco-2 permeability assay was used to determine the permeability of rhodamine and Enniatins. It is reported that when drugs transporting across the Caco-2 monolayer have P_{app} of 14.0×10^{-6} cm/s or more were considered highly permeable, while P_{app} values of less than 5.0×10^{-6} cm/s were characteristic of low permeability (Ma et al., 2014). In this study, the results of rhodamine 123 P_{app} value differed in 12-transwell and 24- transwell. In 24-transwell when 50uM concentration was used rhodamine 123 P_{app} was 0.41± 0.04 $*10^{-5}$ cm/s. In a permeability study, Troutman & Thakker (2003) reported P_{app} for 5-50uM rhodamine 123 as 1.41 * 10^{-6} cm/s. Thus, the P_{app} value obtained from the study is almost 3 times higher than the literature. Similarly, permeability studies for 5uM rhodamine 123 P_{app} were reported between 2,04 – 2.24 $*10^{-6}$ cm/s (Senarathna & Crowe, 2015; Chen et al., 2015). However, when less (5uM, 10uM) concentrations of rhodamine 123 were used in this study, the P_{app} values increased by 22-27 times in both 12-transwell and 24-transwell. One of the aspects that could lead to higher rhodamine 123 permeability could be passage number of cells used in the transport assay. It is reported that lower passage number cells used in the transport assay increases the P_{app} of the rhodamine 123 (S. M. D. K. G. Senarathna & Crowe, 2015). It could be that lower passaged number cell is not fully developed in terms of tight junction or transporters, which might increase

the transportation thus increases permeability. All the P_{app} stated above in the literatures had used the Passage no. between 44-57 for the transport study. However, in this transport study, 50uM rhodamine 123 in 24-transwell was done using passage no of 27-28 (batch 1) while 5uM and 10uM rhodamine 123 transport studies were done using new batch cell of passage no 7-9 (batch 2). Additionally, the clone or strains vary permeability during the transport study (Caro et al. 1995). Since different strain increase in transportation during transport assay as mentioned in chapter 4.2.1. thus, increased permeability. Another aspect could be the use of different plates that had different insert areas as mentioned above in chapter 4.2.1. Since, insert area influences the cumulative permeated amount during transportation thus influences the permeability.

In 12-transwell, ENNB1 exhibited high permeability followed by ENNB, ENNA and ENNA1. ENNB1 P_{app} was 2, 4, and 13 times higher than ENNB, ENNA and ENNA1 respectively. In 24-transwell, P_{app} was highest for ENNA followed by ENNB1 and ENNA1. ENNA1 P_{app} was 2, 2, and 1.5 times higher than ENNA, ENNB and ENNB1 respectively. Nevertheless, P_{app} exhibited from this study shows similarities for ENNA and ENNA1 in both 12-transwell than in 24-transwell except for ENNB and ENNB1 P_{app}. The difference in P_{app} ENNB in 12-transwell and P_{app} ENNB1 in 24-transwell could be explained by the SEM of the replicates. These samples replicates show a significant difference. Nevertheless, the results show that permeability is directly proportion to cumulative permeated amount. Even though cumulative permeated amount increased with the time, P_{app} did not increase with time (appendix 14, 2). Moreover, P_{app} values of ENNs in this study range from 0.43 ± 0.01 - 5.53 ± 0.98 * 10^{-6} cm/s in 12-transwell and 0.63±0.01 - 1.11± 0.03 * 10^{-6} cm/s in 24-transwell indicate that ENNs are low permeable compounds.

4.3. Caco-2 monolayer integrity during transepithelial transport assay

4.3.1. Caco-2 monolayer integrity assessment using TEER.

TEER measurement is used to monitor physiological activities of cell monolayer in *in vitro* in real time (Kannapin et al., 2021). TEER % higher than 100% in all well in 12-transwell shows that 5uM rhodamine 123, 5uM fluorescein, 1uM ENNA, 1uM ENNA1, 1uM ENNB and 1uM ENNB1 did not cause any damage to the cells during the transport assay. However, TEER% of 24-transwell shows that ENNA1 and ENNB (47.77%±3.53 and 68.77%±12.45) caused damaged to the cells during the transport assay while 50uM rhodamine 123, ENNA, ENNB1 and DMSO did not cause damage. Similarly, 5uM rhodamine and 10uM rhodamine did not cause any cell damage in 24-transwell during the transport while comparing to the medium control result. The decrease in TEER could be due to temperature change during measurement or due to unstable TEER measurement (See chapter 5 limitation and recommendation). TEER values measure the resistance of epithelial tissues to paracellular ion flow (Lu et al., 1996). TEER is temperature dependent and performed at constant temperature (Equilibration from 37C to room temperature).

When TEER is performed before equilibration is completed, fluctuate in TEER value occur (Lf et al., 2010). Decreasing TEER value is reported in increase in paracellular permeability (Lf et al., 2010) which proves why rhodamine 123 P_{app} were higher in those Caco-2 seeded transwells. Moreover, rhodamine 123 concentrations used in transport studies in Caco-2 monolayer were not reported to have any damage to the cells in the transport studies (Ahmed et al., 2020; Ma et al., 2014; Troutman & Thakker, 2003; G. Senarathna & Crowe, 2015).

4.3.2. Fluorescein post control assay

Fluorescein is used in transport study as percentages for a cell integrity maker due to its paracellular pathway in the cell (Ahmed et al., 2020). It is stated that fluorescein transport below 1% is considered to have no damage to cells during the transport assay (Ahmed et al., 2020). In this study, fluorescein transport exhibited <1% in the transwell where 5uM rhodamine 123, fluorescein, ENNA, ENNA1, and ENNB were exposed in 12-transwell. This shows that the cells were not damaged during the transport assay and that these compounds were not toxic to the cells. While ENNB1 exposed cell exhibited >1% fluorescein transport showing the damage in the cells. However, looking into the ENNB1 TEER results, it did not cause any damage during the transport assay in 12-transwell. Moreover, it has been reported that ENNB1 is less cytotoxic than ENNA and ENNA1 (Bertero et al., 2020).

5. Limitation and Recommendation

This study aimed to investigate ENNs P_{app} in Caco-2 assay. The results were based on one biological replicate and two technical replicates and there was a large significant difference (SD, SEM) in the results obtained from replicates as well. This varied the results significantly. The variable results obtained in this study could be due to the errors/limitation such as stock solution preparation, calibration solution preparation or pipetting technique that occurred during the transport assay. Therefore, it is recommended to conduct at least 3 biological replicates to have an overview on significant results.

The ENNs binding to plastic might lead to low mass balance which might underestimate P_{app} value while calculation. To overcome this, BSA is recommended to add with transport buffer to the basolateral compartment to increase ENNs extraction efficiency. It is noted that if BSA is added in the donor compartment, the free concentration of the compound might be changed (Hubatsch et al., 2007).

This study results also shows that transportation of ENNs including the positive control rhodamine 123 performed good in 12-transwell compared to 24-transwell. This could be assessed with Caco-2 monolayer integrity assessment done using TEER% where cells in 12-transwell had no damage from the exposure compounds while cells in 24-transwell were damaged by the same exposure compounds. One thing should be highlighted that TEER for 24-transwell were measured by unstable volt Ohm meter that gave unstable electrical resistance. The values were recorded by holding for 10sec. While 12-transwell were measured by stable volt ohm meter and thus gave the stable electrical resistance. Therefore, it is recommended to use of stable ohm meter while assessing electrical resistance. Overall, performing transport assay was much easier in 12-transwell compared to 24-transwell. Most of the transport studies were done in 12-transwell for both ENNs and rhodamine 123. Therefore, there will be more studies to compare (for example more reference P_{app} values for rhodamine 123)

Even though, in this study rhodamine 123 P_{app} was not achieved as stated in the literatures, it is still recommended to use as positive control because rhodamine 123 is a substrate of P-gp and is a good marker to investigate P-gp activity of Caco-2 cell during transport study. Looking at the result of 5uM rhodamine 123 P_{app} in 12-transwell, it had $8.76 \pm 4.28 * 10^{-5}$ cm/s where 1st replicate had P_{app} of $13.04 * 10^{-5}$ cm/s while 2nd replicated had P_{app} of $4.47 * 10^{-5}$ cm/s. If first replicate is taken as an outlier, the 2nd replicate P_{app} falls along with the literatures. Overall, the results show 5uM rhodamine performed good in 12-transwell compared to 24-transwell. Another thing that could have affected the measurement, such as nominal concentration of exposure solution. It is possible that the prepared exposure solution concentration (nominal) might not be the same concentration

(measured). The fluctuate concentration could have impacted the P_{app} values. Thus, it is recommended to add also nominal concentration (time 0min) in the sample. In this study, positive control for the exposure solution was not measured in any transport assays. Therefore, it is important to measure exposure concentration as well to determine the P_{app} accurately. There is also possibility that calibration had error. In this study, rhodamine 123 and fluorescein calibration had one replicate. To overcome this uncertainty, it is recommended to add at least 2 replicates calibrations.

The estimated ENNs P_{app} calculated from Meca et al. (2012) indicates that P_{app} for 1.5uM ENNA, 1.5uM ENNA1, 1.5uM ENNB, and 1.5uM ENNB1 range from 2-2.4 $*10^{-5}$ cm/s at 4 hour of transport, suggesting ENNs a highly permeable compound (appendix 13). On the other hand, this study shows ENNs, a lowly permeable compound. Since, there has been no other study done for ENNs P_{app}, it is recommended to add highly permeable compound such as propranolol as positive control in the transport assay if the efficient quantification method is available. Propranolol has been used as positive control in various transport studies. It exhibits a very high apparent permeability (P_{app}) value of P_{app} $>20 \times 10^{-6}$ cm/s in Caco-2 cell permeability assays (Ahmed et al., 2020).

ENNs mechanism is still unknown, so it is recommended to study transport assay using the enzyme inhibitors for ENNs transporters.

The presence and absence of transport inhibitors are used to study the transport of ENNs in the small intestine. Ivanova et al. (2019) studied the transport of ENNB1 in Caco-2 cell using the inhibitors Verapamil (Ver), MK57, and fumitremorgin C (FTC) for P-gp, MRP2 and BCRP transporters respectively. Same study reported a specific CYP3A4/5 inhibitor troleandomycin (TAO) for the biotransformation of ENNB1 where the formation of both the hydroxylated and the carbonylated metabolites were slowed down in the presence TAO. The other identified inhibitors for the transporters are given in Table 2.

Table 2. Identified transport and enzyme inhibitors of ENNs in the small intestine.

Transporter	Inhibitor	Reference
P-gp	Verapamil, Grapefruit juice, Immunosuppressive agents, SDZ PSC 833, LY335979, GF120918 (GG918), Cyclodextrin, PEG 400, Tween 80 and Cremophor EL	(Estudante et al., 2013; Ivanova et al. 2019)

MRP2	MK571, LTC_4, Phenolphthalein glucuronide, Fluorescein methotrexate, Probenecid, Furosemide, Indomethacin, Grapefruit juice	(Estudante et al., 2013; Ivanova et al. 2019)
BCRP	Estrone, 17-β-estradiol, GG918, Flavonoids, Herb extracts, Gefitinib, Imatinib, Tamoxifen, Novobiocin, Nelfinavir, Ritonavir, Dipyridamole, Fumitremorgin C (FTC) , Ko143, Cyclosporine	(Estudante et al., 2013; Ivanova et al. 2019)
CYP3A4 (Enzyme)	Troleandomycin	Ivanova et al. (2019)

Next to that, adding post fluorescein assay might be a great choice to increase the quality of cell integrity assessment in case TEER shows unstable results as shown in this study.

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Appendix 1: Intestine (metabolism) Enzymes

The most abundant small intestine enzyme that is responsible for the phase I metabolism of the xenobiotics is cytochrome P450 (CYP450). CYP450 is a heme containing protein that play an important role for the bioactivation or detoxification. CYP450 are characterized spectrophotometrically by an intense absorption band at 450 nm in the presence of reduced carbon mono-oxide (Mittal et al., 2015). Only the CYP 1-3 families are responsible for the metabolism of the various xenobiotics (Mittal et al., 2015; Janssen et al., 2020). The most abundant CYP enzyme in the small intestine has been found to be CYP3A4 followed by second abundant CYP2C9 and CYP3A5. The least abundant CYP were CYP2C19, CYP2J2, CYP2D6, and CYP1A1 (Janssen et al., 2020). The CYP refers to the cytochrome P450, first number refers to family, the alphabet afterwards refers to subfamily and the last number refer to gene identifier (Mittal et al., 2015). Xenobiotic can be either inhibitor or inducer of CYP450 enzyme (Mittal et al., 2015). In a normal condition, xenobiotic that enters the intestinal tissue are normally metabolized by CYP450. They are normally broken down into more polar form metabolites through oxidation, reduction, or hydroxylation. These polar metabolites are easy to excrete. However, if xenobiotic is an inducer of CYP450, it increases the metabolism of the xenobiotic, and eventually destroys the chemical. This may not cause the toxicity, but it causes the sub therapeutic effect if the chemical is intended to target any organs or tissues. On the other hand, if a xenobiotic is an inhibitor of CYP450, it blocks the enzyme activity, as a result it decreases the metabolism of xenobiotic. This can cause the toxicity by building up the chemical effects.

Appendix 2: Transport results in 12-transwell with cell

Apparent permeability of compounds in 12-transwell ± SEM.

Time (min)	Average P_{app} (cm/s) 10^-6					
	1uM ENNA	1uM ENNA1	1uM ENNB	1uM ENNB1	5uM Fluorescein	5uM rhodamine 123
30	0.49 ± 0	0	0	0.65±0	1.2 ± 0	87.59 ± 4.28
60	0.88±0.16	0.04±0.02	3.02±1.51	1.65±0.46		
120	1.31± 0.02	0.28±0.01	3.51±0.6	2.33±0.26	1.1 ±0.02	56.49±0.53
180	1.28±0.02	0.43±0.01	5.53±0.99	2.65±0.17	2.4± 0.02	51.12±0.31

Post fluorescein transport

Apparent permeability of compounds in 12-transwell ± SEM.

Samples	P_{app} (cm/s 10^-6)			% amount transport		
	Avg P_{app}	SD	SEM	Avg	SD	SEM
Rho	0.82	0.61	0.43	0.66	0.49	0.35
Flu	0.82	0.07	0.05	0.66	0.05	0.04
ENNA	0.91	0.61	0.43	0.74	0.49	0.35
ENNA1	1.3	0.75	0.53	1.05	0.6	0.43
ENNB	0.91	0.75	0.53	0.74	0.6	0.43
ENNB1	3.12	3.06	2.16	2.52	2.46	1.74

Recovery of ENNs in 12-transwell with cells.

ENNs	12-transwell			24-transwell		
	Avg Recovery	STD	SEM	Avg Recovery	STD	SEM
ENNA	4.20	1.21	2.97	11.62	11.21	7.93
ENNA1	10.17	5.54	7.19	10.78	3.34	2.37
ENNB	15.42	14.08	10.90	0.36		
ENNB1	4.83	1.95	3.41	63.24	19.11	13.51

Appendix 3. Caco-2 cell culture

Materials

1. Culture medium
2. PBS
3. 0.25 Trypsin solution
4. Pipetboy
5. Pipettes (10ml and 5ml)
6. Waste bottle

Working asceptically, tips:

- Place the equipment first, then the warmed medium, and the cells should be placed in the last step
- When removing and adding reagents, the pipette should not touch the cell side of the flask directly to avoid cell damage
- Avoid touching the neck and cap of the flask by hand to avoid contamination
- make sure the pipette remains upside down, a bit higher than working area to avoid contamination.
- always place the bottle/flask's lid facing down while using the bottle. Close the lid first before opening another bottle.

Order: open the lid -> pipette up the chemical-> close the lid-> open the flask's lid-> pipette in the chemical in the flask (at the bottom corner) -> close the lid -> discard the pipette

- pipette in the liquid to avoid bubbles
- remove the bubbles by pipetting just above the liquid
- do not take hand or any materials above the working flask to avoid contamination
- make sure the liquid doesn't touch the flask's neck or cap (it might be contaminated)
- while pipetting do not go above -2 on the pipette scale (For both liquids and bubbles)

Observation of cells

- Take from the CO_2-incubator 1 or more flasks of cells. Look under the microscope to check the cell confluence. Confluence at 50-60% are fit for the cell culturing. The cells may grow unevenly, so check both sides of the flasks and make sure the average confluence is <70%
- When observing the cells, gently shake the flask. Floating cells (dead cells) may be produced especially for young cells and should not be considered in the confluence. If there are too many floating cells and the liquid color changes, remove all the liquid and add another 10 ml CM for further culturing.
- Sub-culturing sould be conducted when the confluence is around 50%-60%.

Methods

1. Pipette all the culture medium out from the flask that contains cells and discard it in a waste bottle.
2. add 6 ml washing media PBS, wash the cells, pipette the PBS from the cells and discard in the waste bottle (PBS will remove the dead cells and serum, serum inhibits the activity of trypsin), repeat twice
3. add 2 ml trypsin make sure it covers the cell layer for approximately 10 seconds, pipette 1 ml of the the trypsin from the cells and discard in waste bottle (trypsin is added for the cell detachment)
4. put the flask in the CO_2-incubator for 5 minutes and check microscopically if all cells are detached afterwards (for a good cell line, <10 mins should be enough for cells to detach, if after an incubation longer than 15 mins, the cells are still attached, it could be because 1: the cells are differentiated to a strong barrier; 2: the cells are growing mutilayers in the flask. Both reasons lead to bad cells)
5. add 5 ml of CM resuspend the cells with the pipette by washing the cell side of the flask 10 times (to collect cells in the medium) and by pipetting up and down 10 times (to separated cell clusters into single cells).
6. pipette 4ml of the cell suspension into a new flask (either discard or use for seeding), leave the remaining 2 ml in the flask or 2 ml in a new flask (the old flask can be used for 3 times). If the original cells are 50-60% confluent, we keep 2mL out of 6 ml, if the original cells are 30% confluent, we keep 4ml out of 6ml. if the original cells are 70-80% confluent, we keep 1ml out of 6ml. make sure the cells are around 20% confluent after subculture. We usually subculture twice per week.
7. pipette the CM in the flask until ±10 ml is reached (so 2ml cells needed + 8 ml CM). Mix it well by moving the flasks up and down, and left and right.
8. Incubate the flask at 37°C in an atmosphere of 5% Co_2.
9. check the cells every day under the microscope to see the confluence for next subculture.

Appendix 4: Caco-2 seeding in 12-transwell and 24-transwell.

Materials

1. Culture medium (CM):
2. PBS
3. 0.25 Trypsin solution
4. Pipetboy
5. Pipettes (10ml and 5ml)
6. Waste bottle
7. Eppendorf (1.5 ml)
8. Plastic tube (50 ml)

Methods

12-transwell

1. Check the cell confluence under the microscope. Use the cells that are >80% confluence for the seeding.
2. Dip the tweezer in the alcohol for 5-10 minutes to sterile). Do this in the hood.
3. Prepare the transwell by inserting the filters in well with the help of sterile tweezer (
4. Pipette 500 ul PBS to apical compartment and then 1500 ul PBS to basolateral compartment. Cover the lib and leave it in the sterile place (hood).
5. Pipette all the culture medium from the flasks out and discard it in a waste bottle.
6. Add 6 ml washing media PBS, wash the cells, pipette the PBS from the cells and discard in the waste bottle (PBS will remove the dead cells and serum, serum inhibits the activity of trypsin), repeat twice
7. Add 2 ml trypsin make sure it covers the cell layer for approximately 10 seconds, pipette 1 ml of the the trypsin from the cells and discard in waste bottle (trypsin is added for the cell detachment)
8. put the flask in the CO_2-incubator for 5 minutes and check microscopically if all cells are detached afterwards (for a good cell line, <10 mins should be enough for cells to detach, if after an incubation longer than 15 mins, the cells are still attached, it could be because 1: the cells are differentiated to a strong barrier; 2: the cells are growing mutilayers in the flask. Both reasons lead to bad cells)
9. Pipette all the tripsinized cell in 50 ml tube.
10. Pipette 0.5 ml trypsinised cells in the eppendorf.
11. Pippet 20 ul trypsinized cell from the eppendorf to the disc.
12. Insert the disc in the cellometer and calculate the total live cell count.
13. Prepare a cell suspension with $4.48*10^5$ cells/ml ($2 * 10^5$ cell/cm^2) (0.5ml; 1.12 cm2 per insert) by adjusting the volume of culture medium to dilute the cell suspension for the transwell.
14. Before the seeding, Aspirate the PBS from the transtranswell, first from the basolateral and then apical.
15. Seed 0.5 ml of cell suspension to the apical chamber and add 1.5 ml culture medium to the basolateral chamber.

- *It is crucial to keep to this order there will be upward pressure when plating the cells if you switch this.*
- Refresh the culture medium each week on Monday, Wednesday and Friday subsequently. Culture medium in both the apical and basolateral sides should be changed.

Transport assays can be performed when cells become confluent, which usually takes 21-22 days post-seeding.

24-transwell

1. Check the cell confluence under the microscope. Use the cells that are >80% confluence for the seeding.
2. Dip the tweezer in the alcohol for 5-10 minutes to sterile). Do this in the hood.
3. Prepare the transwell by inserting the filters in the 2nd and 3rd row of 24 transwell (12 transwell are used) with the help of sterile tweezer.
4. Pipette 100 ul PBS to apical compartment and then 600 ul PBS to basolateral compartment. Cover the lib and leave it in the sterile place (hood).
5. Pipette all the culture medium from the flasks out and discard it in a waste bottle.
6. Add 6 ml washing media PBS, wash the cells, pipette the PBS from the cells and discard in the waste bottle (PBS will remove the dead cells and serum, serum inhibits the activity of trypsin), repeat twice
7. Add 2 ml trypsin make sure it covers the cell layer for approximately 10 seconds, pipette 1 ml of the the trypsin from the cells and discard in waste bottle (trypsin is added for the cell detachment)
8. put the flask in the CO_2-incubator for 5 minutes and check microscopically if all cells are detached afterwards (for a good cell line, <10 mins should be enough for cells to detach, if after an incubation longer than 15 mins, the cells are still attached, it could be because 1: the cells are differentiated to a strong barrier; 2: the cells are growing mutilayers in the flask. Both reasons lead to bad cells)
9. Pipette all the tripsinized cell in 50 ml tube.
10. Pipette 0.5 ml trypsinised cells in the eppendorf.
11. Pippet 20 ul trypsinized cell from the eppendorf to the disc.
12. Insert the disc in the cellometer and calculate the total live cell count.
13. Prepare a cell suspension with $1 * 10^5$ cells/cm^2, $3.3 * 10^5$ cells/ml (0.1ml; 0.33cm2 per insert) by adjusting the volume of culture medium to dilute the cell suspension for the transwell.
14. Before the seeding, Aspirate the PBS from the transtranswell, first from the basolateral and then apical.
15. Seed 100 ul of cell suspension to the apical chamber and add 600 ul culture medium to the basolateral chamber.
    - *It is crucial to keep to this order there will be upward pressure when plating the cells if you switch this.*
    - Refresh the culture medium each week on Monday, Wednesday and Friday subsequently. Culture medium in both the apical and basolateral sides should be changed.
16. Transport assays can be performed when cells become confluent, which usually takes 21-22 days post-seeding.

Appendix 5: plastic binding of ENNs in transwell

In 12-transwell

Day 1- Preparation of exposure medium and extraction solutions:

- Prepare 500 mL acidified acetonitrile by adding 0.5 uL formic acid to 499.5 mL acetonitrile in 500 ml glass beaker with cap in fume hood. Label bottle and store at room temperature.

- In fume hood, prepare 1 mL working stock solution of 800 uM enniatin A in DMSO by adding 80 µL of master stock solution containing 10 mM enniatin A in DMSO to 920 uL DMSO in 1.5 mL glass autosampler vial with screw cap . Vortex for 1 second. Label vial and store at -20C.

- Prepare 1 mL working stock solution of 800 uM enniatin A1 in DMSO by adding 80 µL of master stock solution containing 10 mM enniatin A1 in DMSO to 920 uL DMSO in 1.5 mL glass autosampler vial with screw cap. Vortex for 1 second. Label vial and store at -20C.

- Prepare 1 mL working stock solution of 800 uM enniatin B in DMSO by adding 80 µL of master stock solution containing 10 mM enniatin B in DMSO to 920 uL DMSO in 1.5 mL glass autosampler vial with screw cap. Vortex for 1 second. Label vial and store at -20C.

- Prepare 1 mL working stock solution of 800 uM enniatin B1 in DMSO by adding 80 µL of master stock solution containing 10 mM enniatin B1 in DMSO to 920 uL DMSO in 1.5 mL glass autosampler vial with screw cap. Vortex for 1 second. Label vial and store at -20C.

- Prepare 400 uL dosing stock solution of a mixture of 200 uM enniatin A, A1, B and B1 in DMSO by adding 100 uL working stock solution of 800 uM enniatin A, A1, B, B1 in DMSO together in a 1.5 mL glass autosampler vial. Vortex for 1 second. Store at -20C.

Day 2

- Prepare 12 mL exposure medium with a mixture of 1 uM enniatin A, A1, B and B1 by adding 60 uL dosing stock solution with 200 uM enniatin A, A1, B and B1 in DMSO to 11940 uL exposure medium in a 50 ml amber glass bottle under sterile conditions. Vortex for 3 seconds. Label bottle. Use immediately.

Prepare the 4000 uL working solution of 50 uM Rhodamine 123 in exposure medium in a 4ml amber colored glass vial. For this, add 20 ul mater stock

solution (1) of 10000uM rhodamine 123 in DMSO in a amber glass vial and add 3980 ul transport buffer to reach the final volume of 4000 ul. Cap, vortex 2 seconds and store at room temperature.

Assessing whether enniatins bind problematically to transwell:
- Under sterile conditions, pipet 500 uL exposure medium with a mixture of 1 uM enniatin A, A1, B and B1 into the apical compartment of transwell A1-C1 in a 12-well microtiter plate with transwell inserts.
- Pipet 1500 uL exposure medium with a mixture of 1 uM enniatin A, A1, B and B1 into the basolateral compartment of transwell A1-C1.

- Pipet 500 uL exposure medium to the apical compartment of well C2.

- Pipet 1500 uL exposure medium to the basolateral compartment of well C2.

- Pipet 500 uL Rhodamine with exposure medium to the apical compartment of well A3-B3.
- Pipet 1500 uL Rhodamine with exposure medium to the basolateral compartment of well A3-B3.
- Leave well plate with exposure medium with lid with exposure medium in dark at room temperature 4h for ENNs condition and 3 h for Rhodamine exposure.

-Prepare the standard solution for rhodamine callibration curve.

In 24-transwell

- Prepare the stock solution with the concentration of 800uM test compound by diluting in 100% DMSO in glass vial. It should be taken into account that the exposure media can only contain 0.5% DMSO, therefore the concentration of test compound in the exposure media should be 200 times lower than the concentration of the test compound in DMSO solution. The concentration of test compound in the exposure should be 1uM.

- Prepare the individual exposure solution. For the test compounds, pipette 5 ul stock solution (2) 100% DMSO in the vial. Add 995 ul transport buffer in the vial to reach the final volume of 1600 ul with 0.5%DMSO. Use 1000 ul exposure solution for one technical replicate. Therefore, 1000ul for two technical replicates.
- pipette 5 ul of ENN A, ENN A1, ENN B, and ENN B1 stock solution (1) 800uM, respectively in a amber vial. Add 3980 ul transport buffer in the vial to reach the final volume of 4000 ul to reach 0.5% DMSO.
- Prepare 24 transwell by inserting the filter in the wells.

- Add 100 ul transport medium In apical compartment and 600ul transport medium in basolateral compartment.
- put the transwell plate in incubator for 30mins.
- After 30 min, aspirate all transport medium from basolateral and then apical compartment.
- Expose 100 ul 4 uM mixture ENNS solution in the apical compartment and then 600ul transport medium in the basolateral compartment of transwell.
- Incubated the exposed compound in 5% Co_2 at 37°C for 30 min, 1hr and 2hr.
- At the following exposure time (30mins, 1h), take samples from the basolateral compartment. For this, collect 10ul from the basolateral compartment and add them to an autosampler dark glass vial . vortex and store at -20 °C.
- add in 10ul pre-warmed transport medium to basolateral compartment each time.
- At the final exposure time (2h), take all the liquid from basal compartment
- At the final exposure time (2h), collect all the liquid from apical compartment. vortex and store at -20 °C.
- add 100 ul ACN to the apical of 1 transwell respectively. Add 600 ul ACN to basal compartment of transwell, Put the plate in the shaker at 37 ℃ for 2 hours, Take all solutions from both AP and BL chambers to a vial and mix them well. vortex and store at -20 °C.
- Leave the samples in the room for 2 hours to deforest.
- Measure the sample in LCMS.

Appendix 6: Rhodamine 123 and fluoresceine measurement

Rhodamine 123 standard solution preparation

- Prepare 50 uM rhodamine 123 in transport medium from 10 mM rhodamine 123 in DMSO by adding 399ul transport medium to 2ul 10 mM rhodamine 123 in DMSO in 1.5 ml amber vial. Cap, and vortex 2 sec.
- Prepare 25 uM rhodamine 123 in DMSO by pipetting 100 ul from 50 uM rhodamine 123 in DMSO in a 1.5 ml amber vial and add 100 ul transport medium. Cap, and vortex 2 sec.
- Repeat the dilution till the concentration of 0 as shown in the layout.
- Measure the concentration in the Tecan with excitation of 505nm and emmision of 525nm.

Concentration(uM)	0	0.390625	0.78125	15.625	3.125	6.25	12.5	25	50
	0	100ul* 0.78125uM	100ul* 1.5625uM	100ul* 3.125uM	100ul* 6.25uM	100ul* 12.5uM	100ul* 25uM	100ul* 50uM	2ul*10mM stock
Transport medium(ul)	199	100	100	100	100	100	100	100	399

Fluorescein standard solution preparation

- Prepare 50 uM fluorescein in transport medium from 10 mM fluorescein in DMSO by adding 399ul transport medium to 2ul 10 mM rhodamine 123 in DMSO in 1.5 ml amber vial. Cap, and vortex 2 sec.
- Prepare 25 uM fluorescein in DMSO by pipetting 100 ul from 50 uM fluorescein in DMSO in a 1.5 ml amber vial and add 100 ul transport medium. Cap, and vortex 2 sec.
- Repeat the dilution till the concentration of 0 as shown in the layout.
- Measure the concentration in the Tecan with excitation of 437nm and emmision of 515nm.

Concentration(uM)	0	0.390625	0.78125	15.625	3.125	6.25	12.5	25	50
	0	100ul* 0.78125uM	100ul* 1.5625uM	100ul* 3.125uM	100ul* 6.25uM	100ul* 12.5uM	100ul* 25uM	100ul* 50uM	2ul*10mM stock
Transport medium(ul)	199	100	100	100	100	100	100	100	399

Appendix 7. Transport assay in Caco-2 seeded 12-transwell

Materials

1. Culture medium (CM) stored at 4C. (PK20231127)
2. PBS stored at 4C(1X) Invitrogen cat. No. 10010015
3. 0.25 Trypsin solution stored at 4C
4. Pipetboy
5. Pipettes (10ml and 5ml)
6. Waste bottle
7. Eppendorf (1.5 ml)
8. Plastic tube (50 ml)
9. Rhodamine 123 10 mM stored at -20C (PK20231130)
10. 200uM ENNA in DMSO, 200uM ENNA1 in DMSO, 200uM ENNB in DMSO, 200uM ENNB1 in DMSO (PK20231123)
11. Fluorescein powder stored at room temperature, Lot 052K3470 EC no 2190318
12. 12-well polystyrene plates (Sigma; thincerts Greiner; transtranswell Corning, 12 mm Transwell with 0.4 µm pore polycarbonate membrane insert (Sigma; thincerts Greiner; transtranswell Corning)
13. 96 transwell Greiner 96 Flat Bottom Black Polystyrene Cat. No.: 655090/655096/655097 [GRE96fb_µClear.pdfx]
14. 1.5 mL vial short thread vial 32 x 11.6 mm (amber), wide opening (BGB Analytik Benelux B.V., Germany, Part number: 080401-XL)
15. Transport buffer: Hank's balanced salt solution (HBSS) (without Phenol Red) + 10 mM HEPES, pH 7.4 (1 :100 1 M HEPES in HBSS) stored at 4C.
16. Voltohmmeter
17. 4 ml Amber colored glass vial
18. 25 ml tube

Preparation

- Deforest all the solutions stored at -20C for 1 hour at room temperature.
- Pre-warm all the medium at 37C in the water bath before use.
- label all the amber vials that will be used for the standard solution preparation, and collecting samples at incubation time interval during transport study.

Transport assay

- Prepare a 4000 uL exposure/working solution of 5 uM rhodamine 123 in transport medium in a 1.5 mL amber colored glass vial by adding 3980ul transport medium to 20 ul 1000 uM Rhodamine 123 in DMSO in a amber glass vail. Cap, vortex 2 seconds.
- Prepare a 4000 uL exposure solution of 5 uM fluorescein in transport medium in a 1.5 mL amber colored glass vial by adding 3980ul transport

medium to 20 ul 2000 uM fluorescein in DMSO in a amber glass vail. Cap, vortex 2 seconds.

- Take the Caco-2 seeded 12-transwell and Measure TEER with culture medium using Voltohmmeter.
- Aspirate the culture medium from basal compartment and then apical compartment.
- Add 0.5 ml transport medium to apical compartment, and add 1.5 ml transport medium to basolateral compartment.
- Incubate the transwell at 37C, 5% Co_2 for 25 minutes.
- Measure TEER with transport medium.
- TEER measurement
  - Pippete 20 ml ethanol in 25 ml tube.
  - Pippete 20 ml PBS in 25 ml tube.
  - Pippete 20 ml transport medium in 25 ml tube.
  - Dip the probe in ethanol for 10 minutes.
  - Dip the probe in PBS for 1 minutes.
  - Dip the electrodes in transport medium for 1 minute.
  - Measue TEER by placing longer electrode in the basolateral compartment (let it toucn the compartment). Hold the probe for 20 sec without moving the hand and write down the measurement.
    - Do it for all the transwell.
- Aspirate the transport medium from the basolateral compartment than apical compartment. Be careful with the cell layer.
- Add 0,5 ml 5uM rhodamine 123 in transport medium on apical side of A1 and A2 in 12 transwell.
- Add 1,5 ml transport medium in the basolateral side of A1 and A2 in 12-transwell.
- Add 0,5 ml 5uM fluorescein in transport medium on apical side of A3 and A4 .
- Add 1,5 ml transport medium in the basolateral side of A3 and A4.
- Add 0,5 ml 1 uM ENNA in transport medium on apical side of B1 and C1.
- Add 1,5 ml transport medium in the basolateral side of B1 and C1.
- Add 0,5 ml 1 uM ENNA1 in transport medium on apical side of B2 and C2.
- Add 1,5 ml transport medium in the basolateral side of B2 and C2.
- Add 0,5 ml 1 uM ENNB in transport medium on apical side of B3 and C3.
- Add 1,5 ml transport medium in the basolateral side of B3 and C3.
- Add 0,5 ml 1 uM ENNB1 in transport medium on apical side of B4 and C4.
- Add 1,5 ml transport medium in the basolateral side of B4 and C4.
- Incubate the exposed cells at 37C at 5% CO_2 for 30 min, 1hr, 2hr, and 3 hr.
- After 30 min, collect 100 ul samples from A1-A4 basolateral into a 1.5 ml amber vial from all transwell. Cap, vortex and store at room temperature.
- Add 100 ul transport medium to A1-A4 basolateral.
- After 30 min, collect 50 ul samples from B1-C4 basolateral into a 1.5 ml amber vial from all transwell. Cap, vortex and store at -20C.
- Add 50 ul transport medium to B1-C4 basolateral.

- After 1 hr, collect 50 ul samples from B1-C4 basolateral into a 1.5 ml amber vial from all transwell. Cap, vortex and store at -20C
- Add 50 ul transport medium to B1-C4 basolateral.
- After 2 hr, collect 50 ul samples from B1-C4 basolateral into a 1.5 ml amber vial from all transwell. Cap, vortex and store at -20C.
- Add 50 ul transport medium to B1-C4 basolateral.
- After 2hr, collect 100 ul samples from A1-A4 basolateral into a 1.5 ml amber vial from all transwell. Cap, vortex and store at room temperature.
- Add 100 ul transport medium to A1-A4 basolateral.
- After 3 hr, collect 100 ul samples from A1-A4 basolateral into a 1.5 ml amber vial from all transwell. Cap, vortex and store at room temperature.
- After 3 hr, collect 50 ul samples from B1-C4 basolateral into a 1.5 ml amber vial from all transwell. Cap, vortex and store at -20C.
- After 3 hr, collect 100 ul samples from A1-A4 apical into a 1.5 ml amber vial from all transwell. Cap, vortex and store at room temperature.
- After 3 hr, collect 50 ul samples from B1-C4 apical into a 1.5 ml amber vial from all transwell. Cap, vortex and store at -20C.
- Aspirate the medium from basolateral and then apical from all transwell.
- Pipette 500ul transport medium to apical in each well, and 1500 ul transport medium to basolateral.
- Measure TEER.
- Aspirate all transport medium.
- Pipette 0.5 ml 5uM Fluorescein in DMSO to A1-C4 apical well, and 1500 ul transport medium to A1-C4 basolateral.
- Incubate the exposed cell at 37C at 5% CO_2 for 1 hour.
- While waiting for the incubation time, prepare the standard solution for rhodamine and Fluorescence.
- After 1 hr, collect 100 ul sample from A1-C4 basolateral in 1.5 ml amber vial.
- After 1 hr, collect 100 ul sample from A1-C4 apical in 1.5 ml amber vial.
- Pipette 100ul rhodamine 123 standard solution in 96 transwell according to the layout.
- Pipette 50ul collected samples from A1-A2 at each interval time in the 96 well and add 50 ul transport medium to reach the total volume of 100ul.
- Measure the concentration in the Tecan with excitation of 505nm and emmision of 525nm.
- Pipette 100ul fluorescein standard solution in 96 transwell according to the layout.
- Pipette 50ul collected samples from A3-A4 at each interval time in the 96 well and add 50 ul transport medium to reach the total volume of 100ul.
- Pipette 100ul collected samples from A1-C4 post transport in 96 transwell.
- Measure the concentration in the Tecan with excitation of 437nm and emmision of 515nm.

Appendix 8: Transport assay in Caco-2 seeded 24-transwell.

- Prepare the 4000 uL working solution of 1 uM ENN A in transport buffer in a 4ml amber colored glass vial. For this, add 20 ul minor stock solution of 200uM ENN A in DMSO in a amber glass vial and add 3980 ul transport buffer to reach the final volume of 4000 ul. Repeat this for ENN A1, ENN B, and ENN B1. Cap, vortex 2 seconds and store at room temperature.
- Prepare the 4000 uL working solution of 50 uM Rhodamine 123 in transport buffer in a 4ml amber colored glass vial. For this, add 20 ul mater stock solution (1) of 10000uM ENN A in DMSO in a amber glass vial and add 3980 ul transport buffer to reach the final volume of 4000 ul. Cap, vortex 2 seconds and store at room temperature.
- Take the Caco-2 seeded 24-transwell and aspirate the culture medium from basal compartment and then apical compartment.
- Add 100 ul transport medium to apical compartment, and add 600 ul transport medium to basolateral compartment.
- Incubate the transwell at 37C, 5% Co_2 for 30 minutes.
- Measure TEER in this 30 minutes using Voltohmmeter.
- Aspirate the transport medium from the basolateral compartment than apical compartment. Be careful with the cell layer.
- Add 100ul working solution to the apical compartment and 600 ul transport medium to basolateral compartment. Expose the working solution in two technical replicates according to the layout.
- Incubate the exposed cells at 37C in 5% CO_2 for 30 min, 1 hr, 2 hr, 3hr and 4 hr.
- Collect 30ul sample for ENNs and negative control from the basolateral compartment in a 4 ml amber glass vial. Cap and vortex for 2 sec and store it at -20C.
- Collect 100ul for positive control from the basolateral compartment in a 4 ml amber glass vial. Cap, vortex for 2 sec and store it at room temperature.
- Add 30 ul pre-warmed transport medium to the basolateral to balance the volume in negative control and ENNs.
- Add 100 ul pre-warmed transport medium to the basolateral to balance the volume in positive control .
- Collect 30 ul samples from the basolateral compartment similary at 1, 2 and 3 hour of incubation time.
- While waiting for the two last incubation time, prepare the standard solution for rhodamine 123 calibraton.
- Collect all the sample from the basolateral compartment first and then from apical comoartment at the 4 hours of incubation time.
- Add prewarmed exposure medium to basolateral (600 ul)and apical side (100 ul). Measure TEER to check if the well was affected by the compound. The TEER values of endpoint should at least higher than 80% of beginingpoint to check if the well was affected by the amino acid/bile acid.

- Add 100 ul standard curve solution as shown below layout. Pippete 100ul positive control concentration from basolateral and than apical. Place them in 96 well together with the standard curve solution. Measure the concentration in cellometer with excitation of 505 nm and emmision of 525 nm.

Appendix 9: Recovery calculation

Recovery % of ENNA at 4 hours at room temperature in 12-transwell without cells is calculated below from raw data.

Samples	Concentration measured (nM)	Concentration measured (uM)	Recovery%
A1 Apical EnnMixture and EM 1	101.00	0.10	24.01
B1 Apical EnnMixture and EM 1	70.80	0.07	10.18
C1 Apical EnnMixture and EM 1			16.87
A1 Basolateral EnnMixture and EM 1	66.04	0.07	
B1 Basolateral EnnMixture and EM 1			
C1 Basolateral EnnMixture and EM 1	117.35	0.12	

Positive control 0.25 uM ENNA measured in LCMS = 0,3478167 uM
Recovery % of ENNA replicate 1 on apical compartment = (0.10*0.5/ 0.3478167*0.5) * 100 = 29.04%

Recovery % of ENNA replicate 1 on bsolateral compartment = (0.07*1.5/ 0.3478167*1.5) * 100 = 18.99%

Total recovery % of ENNA replicate 1 = 24.012%

Similarly, all replicates recovery % were calculated and average was taken for the result.
Relative standard deviation (RSD) was calculated to assess whether SD is high or small compared to average recovery.
RSD for ENNA recovery = (SD/Avg recovery) = 9.78/17.02 = 0.57
It means, SD of ENNA recovery is 57% higher than average recovery. The recovery % of ENNA, ENNA1, ENNB, and ENNB1 is shown below table.

Enniatins	Avg Recovery%	STD	RSD
ENNA	17.02	9.78	0.57
ENNA1	65.09	15.21	0.23
ENNB	43.21	10.67	0.25
ENNB1	14.65	2.96	0.20

Appendix 10. TEER

TEER calculation for transport assay in 12-transwell is shown below.

Area of insert = 1.12 cm2
Electrical resistance of blank = 125 Ω

Layout in 12 transwell

	1	2	3	4
A	A1 Rho	A2 Rho	A3 Flu	A4 Flu
B	B1 ENNA	B2 ENNA1	B3 ENNB	B4 ENNB1
C	C1 ENNA	C2 ENNA1	C3 ENNB	C4 ENNB1

Electrical resistance Ω before exposure.

	1	2	3	4
A	517	430	450	472
B	468	485	492	475
C	535	510	488	544

Electrical resistance Ω after exposure.

	1	2	3	4
A	798	717	696	746
B	480	536	770	745
C	845	782	571	730

Equation 1:

$$\text{TEER} = (\text{ER} - \text{blank ER}) \times \text{A}$$

TEER of well A1 before exposure = (517 – 125) * 1.12 = 439.04 Ω × cm2
TEER of well A1 after exposure = (798 – 125) * 1.12 = 753.76 Ω × cm2

TEER %= (753.76/439.04) * 100 = 171.68%

TEER% after the exposure.

Samples	TEER%
Rho	182.89
Flu	177.33
ENNA	139.55
ENNA1	142.41
ENNB	149.31
ENNB1	160.77

Appendix 11. ENNs calibration preparation

Step 1: Begin by combining 25 µL of a dosing stock solution containing a mixture of 200 µM enniatin A, A1, B, and B1 (prepared in DMSO) with 375 µL of exposure medium. Adjust the volume to a final 400 µL, resulting in a concentration of 50 µM.

Step 2: Conduct 15 serial dilutions of ENNs in exposure medium with a dilution factor of 2. Initiate the process by transferring 20 µL of exposure medium to a vial, followed by the addition of 20 µL from the previously prepared 400 µL solution. Thoroughly mix, take 20 µL from the mixed solution, and transfer it to another vial containing 20 µL of exposure medium.

Repeat this serial dilution until the concentration decreases from the initial 50 µM to 0.001526 µM.

Step 2 ENNs dilution in Exposure medium (0.5% DMSO and 99.5% Exposure medium)

	ENNs conc. (uM)	Dillution times	ENNs conc in EM (uM)	Volume ENNs (uL)	Total Volume(uL)	Volume of exposure medium (uL)	%DMSO
1	50	2	25	20	40	20	0.125
2	25	2	12.5	20	40	20	0.0625
3	12.5	2	6.25	20	40	20	0.03125
4	6.25	2	3.125	20	40	20	0.015625
5	3.125	2	1.5625	20	40	20	0.007813
6	1.5625	2	0.78125	20	40	20	0.003906
7	0.78125	2	0.390625	20	40	20	0.001953
8	0.390625	2	0.195313	20	40	20	0.000977
9	0.195313	2	0.097656	20	40	20	0.000488
10	0.097656	2	0.048828	20	40	20	0.000244
11	0.048828	2	0.024414	20	40	20	0.000122
12	0.024414	2	0.012207	20	40	20	6.1E-05
13	0.012207	2	0.006104	20	40	20	3.05E-05
14	0.006104	2	0.003052	20	40	20	1.53E-05
15	0.003052	2	0.001526	20	40	20	7.63E-06

Step 3: Proceed to dilute enniatins in acetonitrile (ACN). The first vial from Step 2 has a concentration of 25 µM. Apply a dilution factor of 4 by taking 15 µL from

the 25 µM solution and adding 45 µL of ACN, resulting in a total volume of 60 µL with a concentration of 6.25 µM.

Continue the dilution process for the second vial, which has a concentration of 12.5 µM after serial dilution. Extract 15 µL from the 12.5 µM vial and combine it with 45 µL of ACN, achieving a total volume of 60 µL with a concentration of 3.125 µM. Repeat these steps, ensuring the concentration ranges from 6.25 µM to 0.000381 µM after the addition of ACN.

Step 3 ENNs dilution in ACN (0.5% DMSO and 99.5% Exposure medium)

ENNs conc. in DMSO and EM (uM)	ENNs conc. 1:3 EM:ACN (uM)	dilution times	Volume ENNs in DMSO and EM (uL)	Total Volume(uL)	Volume of ACN (uL)	%DMSO
25	6.25	4	15	60	45	0.03125
12.5	3.125	4	15	60	45	0.015625
6.25	1.5625	4	15	60	45	0.007813
3.125	0.78125	4	15	60	45	0.003906
1.5625	0.390625	4	15	60	45	0.001953
0.78125	0.195313	4	15	60	45	0.000977
0.390625	0.097656	4	15	60	45	0.000488
0.195313	0.048828	4	15	60	45	0.000244
0.097656	0.024414	4	15	60	45	0.000122
0.048828	0.012207	4	15	60	45	6.1E-05
0.024414	0.006104	4	15	60	45	3.05E-05
0.012207	0.003052	4	15	60	45	1.53E-05
0.006104	0.001526	4	15	60	45	7.63E-06
0.003052	0.000763	4	15	60	45	3.81E-06
0.001526	0.000381	4	15	60	45	1.91E-06

Appendix 12. Apparent permeability

Apparent permeability of 5uM rhodamine 123 in 12-transwell at 180min transport is calculated below using equation 3.

Time (min)	Replicate 1		Replicate 2		Average P_{app} (cm/s) 10^{-5}	P_{app} SD
	Accumulative amount (nmol)	P_{app} (cm/s) 10^{-5}	Accumulative amount (nmol)	P_{app} (cm/s) 10^{-5}		
30	1.31	13.04	0.45	4.48	8.76	6.06
120	2.06	5.12	2.49	6.18	5.65	0.75
180	2.90	4.80	3.28	5.42	5.11	0.44

Q = 2.90 nmol

A = 1.12 cm^2

t = 180 min

C = 5 uM

$$Papp = \frac{Q}{A\ C\ C\ C\ t} = \frac{2.90}{1.12\ C\ 5\ C\ 180\ X\ 60} = 4.8 * 10^{-5}\ cm/s.$$

Appendix 13: Estimation of Enniatins Papp according to Meca et al. (2012).

cell density			12-transwell			Working volume	
250000 cells/cm2			Area	1.12 cm^2		apical	0.5 ml
			diameter	12 mm		basolateral	1.5 ml

Types of ENNs	Compartment	Incubation time (hr)	Exposure concentration (uM)	exposure amount nmol	% measured	transport amount nmol	Papp (cm/s*10^-5)
A	A->B	4	1,5	0,75	76,8	0,576	2,380952
A1	A->B	4	1,5	0,75	70,2	0,5265	2,176339
B	A->B	4	1,5	0,75	67	0,5025	2,077133
B1	A->B	4	1,5	0,75	62,2	0,4665	1,928323
A	A->B	4	3	1,5	57,7	0,8655	1,788814
A1	A->B	4	3	1,5	68,8	1,032	2,132937
B	A->B	4	3	1,5	65	0,975	2,015129
B1	A->B	4	3	1,5	65,1	0,9765	2,018229

Types of ENNs	Compartment	Incubation time (hr)	Exposure concentration (uM)	exposure amount nmol	% measured	transport amount nmol	Papp (cm/s*10^-6)
A	A->B	1	1,5	0,75	20,5	0,15375	6,355407
A1	A->B	1	1,5	0,75	22,5	0,16875	6,975446
B	A->B	1	1,5	0,75	19,5	0,14625	6,045387
B1	A->B	1	1,5	0,75	23,4	0,1755	7,254464
A	A->B	1	3	1,5	24,6	0,369	7,626488
A1	A->B	1	3	1,5	20,7	0,3105	6,417411
B	A->B	1	3	1,5	13,5	0,2025	4,185268
B1	A->B	1	3	1,5	19,4	0,291	6,014385

Appendix 14: Transport of ENNs results in 24-transwell with cell

Apparent permeability of compounds in 24-transwell ± SEM.

Time (min)	Average P_{app} (cm/s) 10^-6				
	ENNA	ENNA1	ENNB	ENNB1	50 uM rhodamines 123
30	1.25 ± 0.02	0.72±0.36	0.6	0.56±0.10	4.13 ± 0.04
60	0.48±0.24	0.06±0.03		0.39±0.19	3.78 ±0.07
120	0.24± 0.12	0.45±0.05		0.31±0.12	2.38±0.01
180	1.11±0.03	0.63±0.01		0.89±0.22	2.87±0.02

Recovery of ENNs in 24-transwell with cell.

ENNs	24-transwell		
	Avg Recovery	STD	SEM
ENNA	11.62	11.21	7.93
ENNA1	10.78	3.34	2.37
ENNB	0.36		
ENNB1	63.24	19.11	13.51