



## A novel “microbiota-host interaction model” to study the real-time effects of fermentation of non-digestible carbohydrate (NDCs) on gut barrier function

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### ABSTRACT

In this study, an *in vitro* co-culture model using an electric cell-substrate impedance sensing system (ECIS) for testing the impact of real-time fermentation of non-digestible carbohydrates (NDCs) by the intestinal microbiota on gut barrier function was established. We applied *Lactobacillus plantarum* WCFS1 as a model intestinal bacterium and alginate-pectin as immobilization polymers as well as a source of NDCs to determine the impact of pectin fermentation on the barrier function of T84 gut epithelial cells. In the first design, *L. plantarum* WCFS1 was encapsulated in an alginate capsule followed by embedding in an agar layer to mimic a firm mucus layer that might be present in the colon. In this experimental design, the presence of the agar layer interfered with the transepithelial electrical resistance (TEER) measurement of T84 cells. Subsequently, we removed the agar layer and used encapsulated bacteria in an alginate gel and found that the TEER measurement was adequate. The encapsulation of the *L. plantarum* WCFS1 does avoid direct contact with cells. Also, the encapsulation system allows higher amounts of packing densities of *L. plantarum* WCFS1 in a limited space which can limit the oxygen concentration within the capsule and therefore create anaerobic conditions. To test this design, T84 cells were co-incubated with *L. plantarum* alginate-capsules supplemented with graded loads of fermentable pectin (0, 4, and 8 mg/ml per capsule) to investigate the effect of pectin fermentation on gut barrier function. We observed that as the pectin content in the *L. plantarum* capsules increased, pectin showed a gradually stronger protective effect on the TEER of the gut epithelium. This could partly be explained by enhanced SCFA production as both lactate and acetate were enhanced in *L. plantarum* containing alginate capsules with 8 mg/ml pectin. Overall, this newly designed *in vitro* co-culture model allows for studying the impact of bacteria-derived fermentation products but also for studying the direct effects of NDCs on gut barrier function in a relatively high-throughput way.

### 1. Introduction

Over the past few decades, scientific evidence for the beneficial effects of non-digestible carbohydrates (NDCs) on gut health has been accumulating (Chanmuang et al., 2022). The gut barrier is the gatekeeper of the body and its function plays a vital role in gut health (Farhadi et al., 2003; Figueroa-Lozano and de Vos, 2019). The gut barrier is a multi-layered functional unit, consisting of gut microbiota, the mucus layer containing antimicrobial proteins, the epithelial cells,

and the lamina propria containing immune cells (Viggiano et al., 2015). NDCs can directly interact with pattern recognition receptors on epithelial cells and immune cells to stimulate mucus secretion and strengthen the gut barrier in a microbiota-independent manner (Cai et al., 2020; Chen and de Vos, 2023). However, recently, more studies have confirmed that not only the direct effect of NDCs on gut epithelial and immune cells is responsible for the improvement of the intestinal immune barrier, but also the beneficial effects of its microbial fermentation products (Chen and de Vos, 2023). NDCs can after intake escape

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from digestion in the upper digestive tract and enter the large intestine (Louis et al., 2007). In the large intestine, a large amount of gut microbiota is colonized which has a symbiotic relationship with the human gut (Heiss and Olofsson, 2018; Pham et al., 2018). The gut microbiota plays a fundamental role in regulating the gut-immune system (Pham et al., 2018). NDCs can be fermented by the gut microbiota and transformed into microbial metabolites such as short-chain fatty acids (SCFAs). These SCFAs can bind to G protein-coupled receptors on the epithelial cells and immune cells to facilitate the development of the intestinal barrier (Chanmuang et al., 2022).

The direct beneficial effects of NDCs on the intestinal barrier have been extensively studied (Chen and de Vos, 2023). However, there is still a lack of *in vitro* co-culture models to study the effects of microbial fermentation of NDCs on gut barrier function. The main challenge for building such a model is that the oxygen levels gradually increase from the lumen to the lamina propria which is hard to mimic *in vitro*. The gut microbiota is anaerobic while the epithelial cells side is aerobic (Sardelli et al., 2021). Besides, gut microbial fermentation is a dynamic process, the metabolites produced at different time points vary and are different in composition over time (Deroover et al., 2017). A previous study has demonstrated the effect of metabolites produced by gut microbiota on gut immune barrier function. However, in this study, the fermentation metabolites were collected at fixed time points to stimulate dendritic cells (Akkerman, 2021). This study showed that the composition of metabolites produced at different fermentation time points varied considerably and that such an approach of testing only at fixed time points is not fully representative of the dynamic process of microbial fermentation and its impact on gut barrier function. Therefore, an *in vitro* model where epithelial cells are continuously exposed to different and dynamic processes of NDC fermentation is needed to get a real predictive and representative view of microbe-host epithelial interactions.

In this study, we aimed to build a real-time fermentation co-culture model for testing NDCs fermentation on gut barrier function. We used *Lactobacillus plantarum*, a commensal resident of the human gut, as a model organism and pectin was used as a source of NDC for following the impact of fermentation. To this end, *L. plantarum* was encapsulated in an alginate capsule supplemented with graded loads of fermentable pectin and embedded in the absence or presence of an agar layer. Then they were cocultured with the gut epithelial cells. Firstly, we tested whether gut epithelial cells could maintain transepithelial electrical resistance (TEER) and remain sensitive to a disruptor of the barrier in this newly designed system. We subsequently selected the best-performing system. Secondly, we investigated whether alginate-pectin fermentation can prevent barrier disruption in this selected system and analyzed the metabolites produced.

## 2. Materials and methods

### 2.1. Experimental design

A real-time, dynamic fermentation co-culture model for testing the impact of NDCs fermentation on gut barrier function was established in a 96-well plate with gold electrodes at the bottom. The upper side of the design was developed to mimic NDCs fermentation by bacteria or by, for example probiotic species, and allows exposure of the epithelial cells for longer times to the different microbial metabolites that are formed during the fermentation of NDCs. A model organism, *L. plantarum WCFS1*, was encapsulated in a high-guluronic acid (G) alginate capsule supplemented with graded loads of fermentable pectin (0, 4, and 8 mg/ml per capsule). The encapsulation of the *L. plantarum WCFS1* does avoid direct contact with the gut epithelium just like in the intestinal area. Another advantage of the system is that packing higher amounts of *L. plantarum WCFS1* which is a facultative anaerobic strain and growing large amounts of *L. plantarum* in a limited space can limit the oxygen concentration within the capsule. This allows anaerobic fermentation in

the core of the capsule. These capsules with bacteria were tested in this system (Fig. 1A). In another separate design, *L. plantarum WCFS1*-containing capsules were embedded in a 0.5 cm thick agar layer to mimic the large intestinal epithelial layer more closely. The colonic epithelium is covered with a firm inner mucus layer and a loose outer mucus layer (Fang et al., 2021) (Fig. 1B). At the bottom of the plates, intestinal epithelial T84 cells were seeded and grown to a differentiated monolayer for 21 days. During the co-culture of the upper and lower sides, a real-time electric cell-substrate impedance sensing system (ECIS) was used to measure TEER to quantify how microbial fermentation influences the gut barrier. The TEER was continuously measured at multiple frequencies of 400 Hz after different challenges. To test the system for protective effects on gut barrier function, T84 cells were challenged with a gut barrier disruptor, i.e. the calcium ionophores A23187, which increases intracellular calcium (Fernández-Lainez et al., 2022). Firstly, to test whether T84 cells can maintain TEER and remain sensitive to the barrier disruptor in this newly designed coculture system, the agar with encapsulated *L. plantarum WCFS1* was pre-incubated with the T84 cells for 12 h, followed by the addition of 4  $\mu$ M A23187. Secondly, to test whether the presence of the agar layer interferes with TEER measurements, only encapsulated *L. plantarum WCFS1* were co-cultured with T84 cells for 12 h followed by A23187 stimulation. Finally, to test whether alginate-pectin fermentation can prevent T84 gut epithelial cells against A23187-induced loss of TEER we added and compared TEER after the addition of 4 and 8 mg/ml pectin to *L. plantarum WCFS1*-containing capsules. The supernatant was collected to determine the production of SCFAs and other intermediate organic acids.

### 2.2. Bacterial cultures

*Lactobacillus plantarum WCFS1* from glycerol stock stored at  $-80^{\circ}\text{C}$  was streaked onto a De Man-Rogosa-Sharpe (MRS) agar (Sigma-Aldrich, St. Louis, MO USA) plate. After 24 h culture at  $37^{\circ}\text{C}$ , one single colony from the plate was inoculated to 10 ml MRS broth (Merck, Darmstadt, Germany) for 12 h culture at  $37^{\circ}\text{C}$ . The OD<sub>600</sub> of the cultured *L. plantarum WCFS1* was measured and the CFU was subsequently adjusted to obtain  $2.5\text{--}3 \times 10^9$  CFU/ml *L. plantarum WCFS1* pellets.

### 2.3. Encapsulation of *L. plantarum WCFS1* in alginate with graded loads of fermentable pectin

The capsules were made of 1.9% (w/v) alginate and supplemented with 0, 4, and 8 mg/ml fermentable pectin. To this end, we used a 1.9% (w/v) high guluronic acid (G) alginate (>50% G) (Manugel® DMB, Tadworth, UK) solution in  $\text{Ca}^{2+}$ -free Krebs-Ringer-Hepes (KRH) buffer with an appropriate osmolarity. As a pectin source, we used a low degree of methyl-esterification (DM = 25) and a high degree of blockiness (DB) orange pectin. Both the alginate and pectin were overnight sterilized by ultraviolet irradiation and then were separately dissolved in 100 ml  $\text{Ca}^{2+}$ -free KRH (220 mOsm) solution under gentle stirring in the concentrations indicated above. After mixing, the solutions were centrifuged for 3 min at 1200 rpm to remove bubbles from the solutions before transforming them into beads.

Before encapsulation 500  $\mu$ L cultured *L. plantarum WCFS1* was first washed with 1.5 ml KRH buffer containing 2.5 mM of  $\text{CaCl}_2$  and centrifuged at 4000 rpm for 10 min. Then the supernatant was removed and the *L. plantarum WCFS1* pellets were collected. To thoroughly mix the *L. plantarum WCFS1* pellets with the alginate/pectin solution, the *L. plantarum WCFS1* pellets were mixed with 400  $\mu$ L KRH buffer containing 2.5 mM of  $\text{CaCl}_2$ . Then the *L. plantarum WCFS1* pellets suspension was mixed with 1600  $\mu$ L alginate/pectin solution to a final volume of 2 ml. This method of mixing in a ratio of 1:4 was derived from Mahmoud et al., (2020).

Transforming the alginate/pectin solutions into capsules was done according to an extrusion technique (Mahmoud et al., 2020). The mixture was extruded through 2 ml sterile syringes with 25 G needles

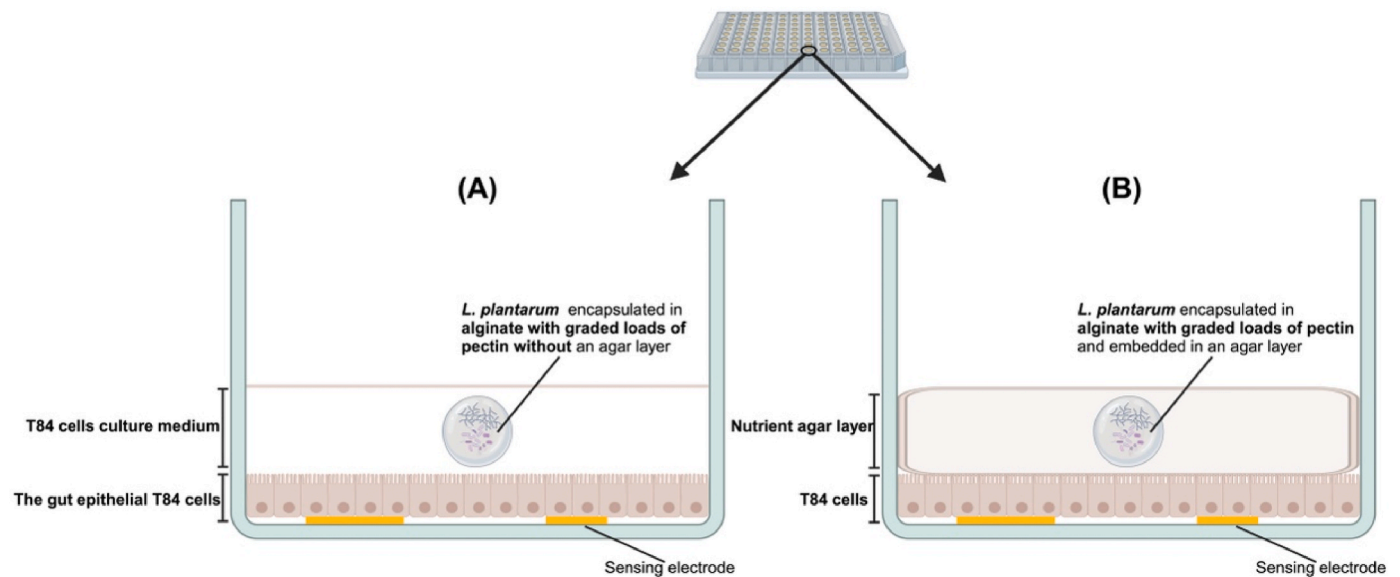


Fig. 1. The experimental design and setup for testing gut barrier function in the presence of encapsulated *L. plantarum* WCFS1 exposed to graded loads of pectin and embedded in the absence (A) or presence of an agar layer (B).

and dropped into a gelification solution (100 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) resulting in the formation of the capsules with a diameter ranging from 2.4 to 2.5 mm. Each capsule contained approximately  $5\text{--}7.5 \times 10^6$  CFUs of *L. plantarum* WCFS1. This was determined by dissolving one capsule in 2 ml of 2% (w/v) tri-sodium citrate solution followed by standard plate counting. Before the capsules were embedded into the nutrient agar or directly co-cultured with T84 cells, the capsules were washed three times with KRH buffer containing 2.5 mM of  $\text{CaCl}_2$ .

#### 2.4. Embedding *L. plantarum* WCFS1-containing capsules in an agar layer

The agar was composed of MRS broth supplemented with 1.5% (w/v) select agar (Sigma-Aldrich, St. Louis, MO USA). After autoclaving, it was stored in a stove at  $55^\circ\text{C}$  and kept in a liquid state. The procedure of embedding *L. plantarum* WCFS1-containing capsules in an agar layer is illustrated in Fig. 2. Firstly, capsules were placed in a  $35 \times 10$  mm petri dish, and 4.4 ml of liquid agar was added, which was subsequently allowed to cool down and become solid to form a layer of agar with *L. plantarum* WCFS1-containing capsules. The final agar layer was always 0.5 cm thick (Fig. 2A). Then, the agar layer in the petri dish was cut by the reverse side of a sterilized 200  $\mu\text{L}$  pipette tip. In this way, the agar was tailored to a size that did fit perfectly into the 96-well plate for subsequent co-culture with T84 cells. During the process of cutting, we ensured that each *L. plantarum* WCFS1 capsule was embedded in the center of the agar layer (Fig. 2B). We subsequently placed the capsule

containing 200  $\mu\text{L}$  pipette tip on a 5 ml pipet which was connected to a pipetboy (Integra biosciences<sup>TM</sup>, Cergy Pontoise, France) and was gently brought by expiration into the 96-well plate containing the T84-cells (Fig. 2C).

#### 2.5. T84 cell culture

The human colorectal carcinoma epithelial cell lines T84 (Sigma-Aldrich, Zwijndrecht, The Netherlands) between passages 19–25 were applied in this study. T84 cells were maintained at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  and cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco, Grand Island, NY USA) supplemented with 10% heat-deactivated fetal bovine serum (Serana Europe GmbH, Pessin, Germany), 15 mM HEPES buffer solution (Gibco, Paisley, UK) and 60  $\mu\text{g}/\text{ml}$  gentamicin solution (Capricorn Scientific GmbH, Ebsdorfergrund, Germany). The culture medium was refreshed every other day. When the T84 cells were grown to 80% confluency, cells were passaged after treatment with 0.1% trypsin-EDTA (Gibco, Grand Island, NY USA).

#### 2.6. Trans-epithelial electrical resistance (TEER) measurement

A 96-well PET plate containing gold electrodes at the bottom (96W20idf PET, Applied Biophysics) was first treated with 300  $\mu\text{L}$  per well of a 2 mg/ml L-cysteine (Merck, Darmstadt, Germany) solution in PBS for 30 min at room temperature. Afterward, the plates were washed with Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L

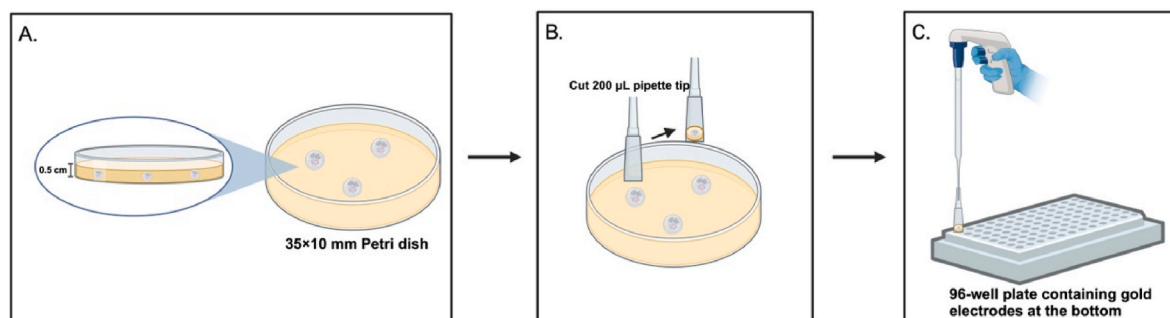


Fig. 2. The procedure of embedding *L. plantarum* WCFS1-containing capsules in an agar layer and placement in an epithelial cell containing 96-well plate.

glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate (Gibco, Grand Island, NY USA) and coated overnight at room temperature with 300  $\mu$ L per well 1% type I bovine collagen (PureCol®, Advanced BioMatrix, San Diego, CA USA) and 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO USA) in DMEM. Plates were then washed twice with complete medium, and T84 cells were seeded at a density of 10,000 cells per well at a final culture volume of 300  $\mu$ L per well and cultured at 37 °C with 5% CO<sub>2</sub> for 21 days to form a gut monolayer with stable TEER. The culture medium was changed every other day. The TEER of the T84 monolayer was continuously measured in a real-time electric cell-substrate impedance sensing system (ECIS, Applied BioPhysics™ model Z0, Troy, NY USA) at multiple frequencies of 400 Hz. Before the experiment started, the plates were placed in the ECIS equipment, and the resistance was monitored overnight to confirm the stability of the TEER. During the experiment, the T84 cells were pre-incubated with *L. plantarum WCFS1* encapsulated in alginate with graded loads of pectin (0, 4, and 8 mg/ml) and embedded in or not in an agar layer for 12 h. Subsequently, the gut barrier disruptor, calcium ionophores A23187 (4  $\mu$ M, Sigma-Aldrich, St. Louis, MO USA) was added, and the TEER was monitored for another 12 h during the presence of A23187. The T84 cells incubated with complete medium only were used as untreated control. The T84 cells treated with A23187 only were used as positive controls. Each group had six technical replicates and this experiment was performed at least five times. To quantify the TEER changes after the treatment with A23187 under different experimental conditions, the area under the curve (AUC) was calculated.

#### 2.7. Measurement of short-chain fatty acids (SCFAs) and other intermediate organic acids

The supernatants for the organic acid analyses were taken after 12 h of A23187 treatment. The samples were centrifuged at 15,000 g for 10 min and 800  $\mu$ L of the supernatant in each sample was collected in an Eppendorf Safe lock® tube. The supernatant was heated at 100 °C for 5 min to inactivate possible enzymes in the samples and stored at -20 °C until further analysis. To quantify the production of SCFAs and organic acids, the samples were subjected to High-Performance Liquid Chromatography (HPLC) analysis as described elsewhere with minor modifications (Beukema et al., 2021). The supernatant (10  $\mu$ L of injection volume) was analyzed to measure organic acids using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, California, USA) with an Aminex HPX-87H column (Bio-Rad laboratories Inc., Hercules, USA). The analyzed SCFA included acetate, propionate, and butyrate as well as the organic acids lactate and succinate were detected with a refractive index detector (RI-101, Shodex, Yokohama, Japan). The samples were eluted in 50 mM sulphuric acid at a flow rate of 0.6 ml/min at 40 °C. The standard curve was prepared in a range of 0.5–3.5 mg/ml for quantification. Data analysis was performed with Chormeleon™ 7.3.1 software from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

#### 2.8. Statistical analysis

Prism 10.0.2 software (GraphPad) was used to perform the statistical analysis. Normal distribution of data was confirmed with the D'Agostino & Pearson test. The results that were normally distributed were expressed as mean  $\pm$  SEM. The statistical significance between treatments and controls was evaluated by one-way ANOVA with Holm-Šidák's multiple comparisons. A *p*-value <0.05 was considered statically significant, # or \**p* < 0.05, ## or \*\**p* < 0.01, ### or \*\*\**p* < 0.001, and #### or \*\*\*\**p* < 0.0001.

### 3. Results

#### 3.1. The agar layer interfered with adequate measurement of the disruption and lowered the TEER of T84 gut epithelial cell monolayers

Fig. 1 illustrates the two systems we designed to follow the impact of fermentation of alginate-pectin as dietary fibers on the barrier (dys) function in real time on monolayers of T84 cells. We first tested the system containing a layer of agar on top of the T84 cells as this system mimics the colonic situation in which gut epithelial cells are covered by a firm mucus layer (Fang et al., 2021). After many different experimental prototypes in which we produced agar layers from different concentrations and thicknesses, we found that an agar concentration of 1.5% (w/v) with a thickness of 0.5 cm was strong enough to withstand the shear forces we needed to apply to place the gel in a 96-well plate.

Next, we tested whether T84 cell monolayers can maintain TEER and remain sensitive to barrier disruptors in this newly designed system. To this end, epithelial T84 cells were pre-incubated with an agar layer containing encapsulated *L. plantarum WCFS1* before the addition of the barrier stressor calcium ionophore A23187 after which the TEER was measured for 12 h. To study the impact of the agar layers as such we also tested T84 cells with the agar on top without the encapsulated *L. plantarum WCFS1*. Also, in these wells, we added A23187 and followed TEER. Fig. 3A shows a representative sample of the relative TEER values compared to the untreated control after adding the barrier disruptor A23187. To calculate the AUC a 12-h interval was used. As shown in Fig. 3B, the incubation of T84 cells with A23187 without the agar layer for 12 h significantly decreased the AUC by 19.16% (*p* < 0.05) compared with that of the untreated control. This was different in the presence of the agar layer. With the agar layer, the AUC values decreased after A23187 administration only by 9.04% and 6.40% for T84 cell cocultures with the agar only and the agar containing *L. plantarum WCFS1* capsule respectively. Even after nine repetitions, this did not reach statistical significance.

Based on these results, we considered that the presence of the agar layer might prevent direct contact of A23187 with the intestinal epithelial barrier. This consideration was based on the fact that we did not observe a prompt decrease in TEER upon the addition of A23187 (Fig. 3A). To determine whether this was the cause of the lack of effect of A23187, we performed an experiment in which a small hole was punched in the middle of the agar layer by a needle. After that, the calcium carrier A23187 was injected directly into the epithelium through this hole by a micro syringe. Before this direct injection of A23187, the epithelial T84 cells were pre-incubated with an agar layer with and without encapsulated *L. plantarum WCFS1* after which the TEER was measured for 12 h. Fig. 4 shows a representative sample of the relative TEER values compared to the controls after injecting the barrier disruptor A23187. However, even with direct injection, we found a severe delay in the drop in TEER in T84 monolayers with agar on top. These results suggest that the presence of agar as such may have a strong effect on the TEER of T84 cells.

Thus, in the next setup, we decided to remove the agar layer from the system and study the impact of the encapsulated *L. plantarum WCFS1* only on the T84 cell barrier function. The experiment was performed as in the previous design where T84 cells were exposed to A23187 for 12 h. Fig. 5A shows a representative sample of the relative TEER values compared to that of the untreated controls after the addition of the barrier disruptor A23187. As shown in Fig. 5B, in the absence of the capsules with *L. plantarum WCFS1* the incubation of T84 cells with A23187 for 12 h significantly decreased the AUC by 19.16% (*p* < 0.0001) compared with that of the untreated control. Also, in the presence of the encapsulated *L. plantarum WCFS1* we found a significant impact of A23187 and found a reduction of 64.69% (*p* < 0.0001). This finding confirms that the agar layer interferes with the effects of calcium ionophore A23187 on the T84 barrier function.

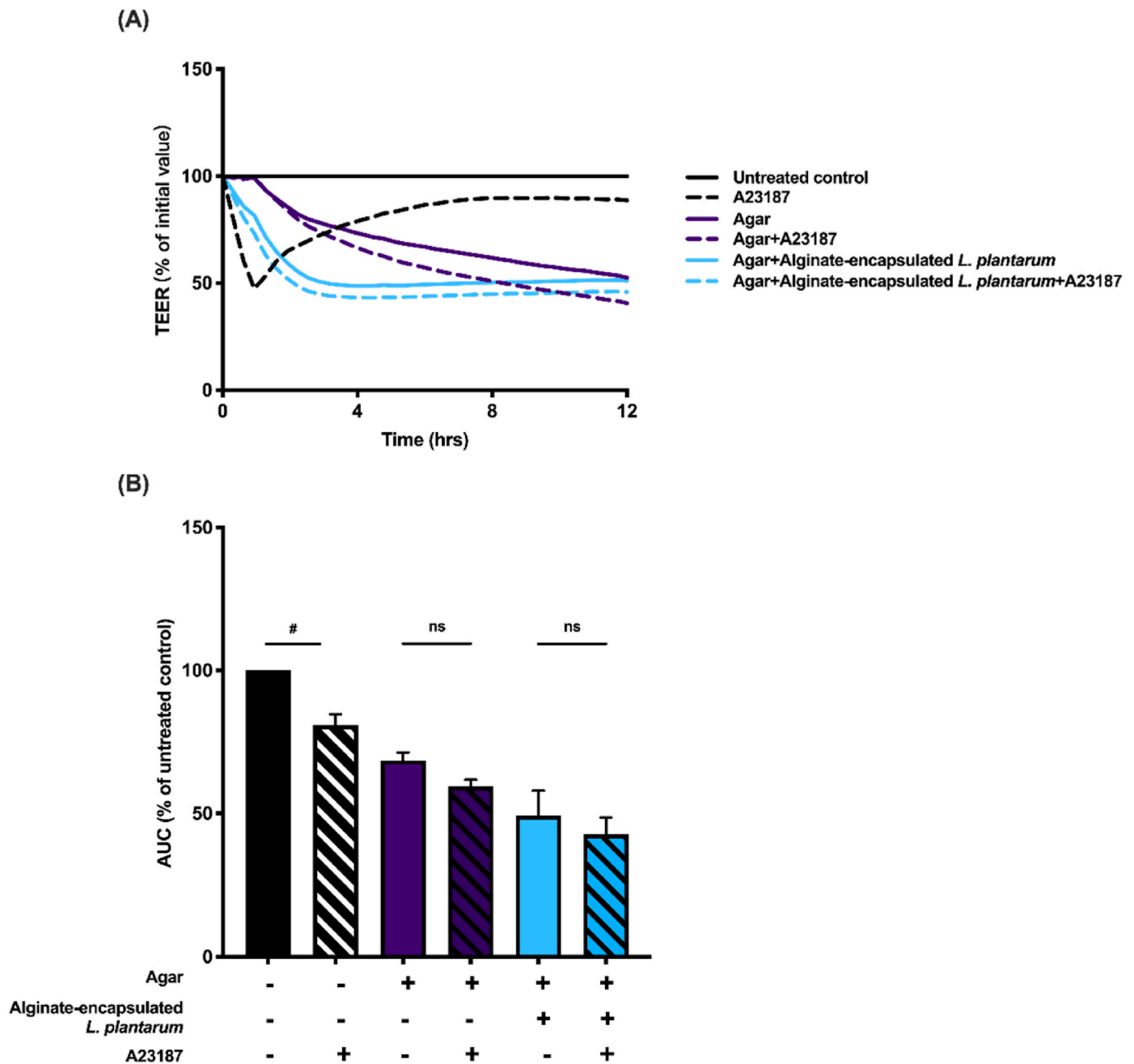


Fig. 3. Barrier disruption induced by A23187 in T84 monolayer when cocultured with an agar layer on top of the cells or with an agar layer containing encapsulated *L. plantarum* WCFS1. The T84 cells were pre-incubated with the agar layer in the presence or absence of encapsulated *L. plantarum* WCFS1 for 12 h. Afterward, the calcium ionophore A23187 (4  $\mu$ M) was added, and the T84 cells were incubated for another 12 h. (A). A representative example of TEER measurement with ECIS after the addition of the barrier disruptor A23187 in T84 cells cocultured with an agar layer containing or not containing encapsulated *L. plantarum* WCFS1. (B). The calculated area under the curve (AUC). The AUC of T84 cells without A23187 treatment was set as 100%. The % AUC of every experimental condition was calculated based on the untreated control. Statistical differences between different groups with the addition of A23187 treatment were compared with those without A23187. Data from nine independent experiments are presented as means  $\pm$  SEM. # $p$  < 0.05.

### 3.2. Pectin-alginate fermentation by encapsulated *L. plantarum* WCFS1 has a protective effect on calcium ionophore-induced disruption of gut epithelial cells barrier function

Next, we determined whether the inclusion of graded loads of pectin in the capsules can prevent the impact of the calcium ionophore A23187-induced barrier disruption. To determine whether this was caused by fermentation of the alginate-pectin by *L. plantarum* WCFS1 and not by the capsules as such, we first test whether the pectin in the alginate capsules without the *L. plantarum* WCFS1 has any direct

protective effects on A23187-induced T84 cells disruption.

Fig. 6A shows the change in relative TEER values after the addition of A23187 compared to the T84 cells pre-cultured with alginate capsules without *L. plantarum* WCFS1 and pectin. Fig. 6B and C illustrate the calculated AUCs. The addition of pectin did not influence the AUC of T84 cells in the absence of A23187 (Fig. 6B). Similar results were observed with the addition of A23187. A23187 disrupted the TEER as the AUC significantly decreased by 53.40% ( $p$  < 0.05), but the increase in pectin content of alginate capsules did not have any protective effect on the barrier function of T84 cells (Fig. 6C).

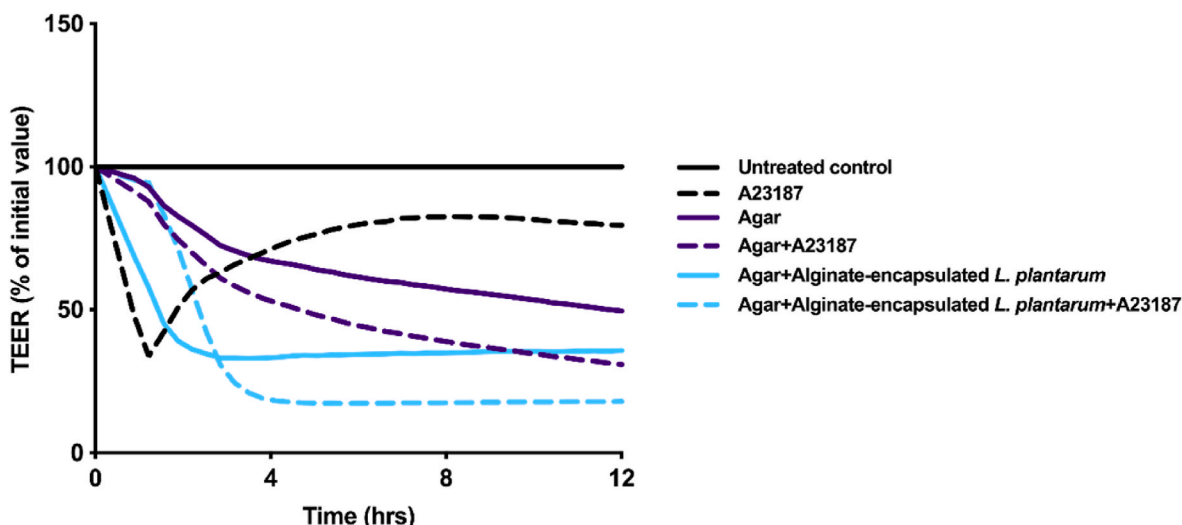


Fig. 4. A representative example of TEER measurement with ECIS after the injection of the barrier disruptor A23187 through the agar layer to the T84 monolayers. Before the injection of A23187, the T84 cells were pre-incubated with the agar layer in the absence or presence of encapsulated *L. plantarum* WCFS1 for 12 h.

Next, T84 cells were incubated with alginate-encapsulated *L. plantarum* WCFS1 containing graded loads of pectin (0, 4, and 8 mg/ml per capsule) followed by a 12-h treatment with A23187. Organic acid production in the culture medium was also determined. Fig. 7A shows the relative TEER values change after the addition of the A23187 compared with the group that T84 cells pre-incubated with the *L. plantarum* WCFS1 alginate capsules without pectin. This allowed us to determine whether the addition of higher amounts of pectin would stimulate fermentation. Fig. 7B and C show the AUCs. There were no effects of pectin addition on AUC in the absence of A23187 (Fig. 7B). This was different in the presence of the A23187 (Fig. 7C). After adding the barrier disruptor A23187, we found a rescuing effect of pectin addition in the capsules on A23187-induced barrier disruption and this effect was pectin concentration dependent. With 4 mg/ml of pectin, we did not find any statistically significant rescuing effect of pectin on A23187-induced barrier disruption, even though the AUC increased by 5.83% compared to T84-cells exposed to A23187 in the absence of pectin. This was different at the higher 8 mg/ml pectin concentration. Here the rescuing effect of the A23187-induced T84 barrier disruption was 21.15% ( $p < 0.001$ ) compared to the group without pectin. Also, it was 15.32% ( $p < 0.01$ ) higher and statistically significantly different from the group exposed to 4 mg/ml of pectin.

We next tested for the presence of SCFAs in the supernatants, to determine whether the effect of adding graded loads of pectin to the capsules and its enhancing rescuing effect on A23187-induced barrier disruption can be attributed to the fermentation of pectin or to direct effects of the pectin on the epithelial cells. We only detected lactate and acetate. The levels of are shown in Fig. 8. In the supernatant *L. plantarum* in alginate only capsules already induced significant production of lactate which was not further enhanced by the addition of pectin. Only at 8 mg/ml of pectin we found a minor and not statistically significant enhancement of 0.038 mg/ml compared to *L. plantarum* in alginate capsules ( $p = 0.38$ ) (Fig. 8A). The acetate production results were different. The addition of the stressor lowered the acetate production which remained low when 4 mg/ml of pectin was present in the capsules but enhanced 0.56 mg/ml production ( $p = 0.13$ ) when 8 mg/ml of pectin was added (Fig. 8B).

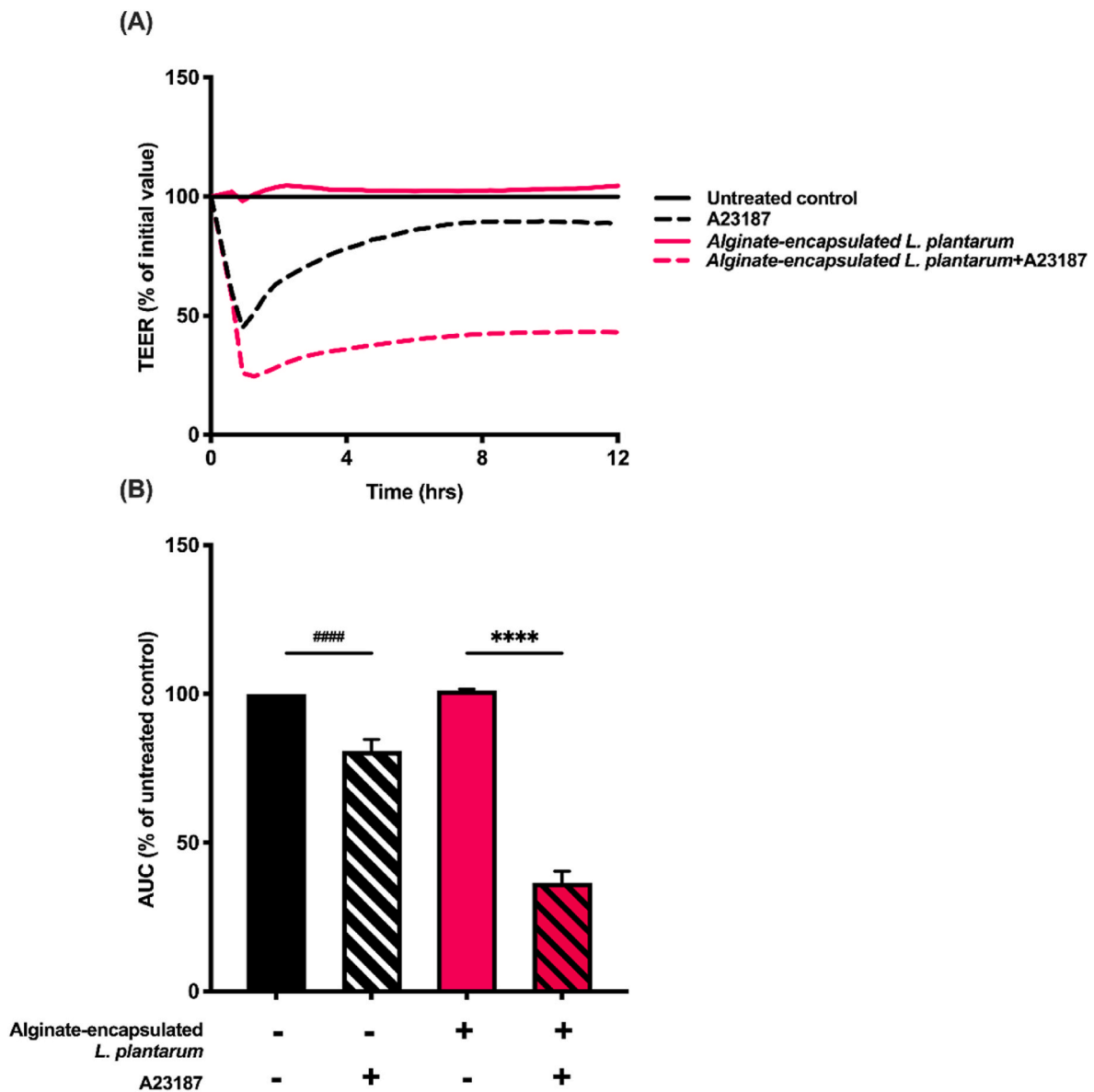
#### 4. Discussion

In recent years, scientific evidence has become available from *in vitro* studies that demonstrate beneficial effects of NDC-derived microbial fermentation products on gut-immune barrier function (Akkerman,

2021; Van den Abbeele et al., 2020). However, these fermentation products were always collected at fixed time points followed by the stimulation of gut epithelial cells or immune cells (Akkerman, 2021; Van den Abbeele et al., 2020). These studies, therefore, do not fully mimic the dynamic process of fermentation and its possible impact on gut barrier function. It was, however, far from easy to create such a system that dynamically follows barrier function during fermentation by coculturing bacteria and epithelial cells together. The main reason for this is the oxygen gradient along the radial axis that is needed to allow anaerobic fermentation. The gut microbiota is mostly anaerobic while the gut epithelia need an aerobic environment (Sardelli et al., 2021).

In this study, we developed and characterized a real-time fermentation co-culture model for testing NDCs fermentation on gut barrier function. We used facultative anaerobe *L. plantarum* as a model organism. It was encapsulated in alginate capsules followed by embedding in or without an agar layer before directly co-cultivating with T84 epithelial cells. The TEER of the intestinal barrier was continuously monitored in the ECIS system. Unique features compared to other existing modeling systems include: (I) Encapsulation avoids direct contact of *L. plantarum* with the T84 epithelium allowing study of impact of fermentation products only in the absence of contact of the bacteria with the cells. (II) *L. plantarum* is in a limited space and after a period of growth tends to create an anaerobic environment in the capsule allowing anaerobic fermentation. (III) Metabolite profile can be determined by collecting supernatants. (IV). By doing this design in the ECIS system we can continuously measure T84 epithelial TEER which allows monitoring of changes in intestinal epithelial TEER due to dynamic fermentation of NDCs. Another advantage is that the TEER measurement of the ECIS system is more accurate than that of the commonly used transwell co-culture system (Srinivasan et al., 2015; Szulcek et al., 2014).

Initially, we included an agar layer between the bacteria and epithelial cells to mimic the presence of a mucus layer. However, the presence of the agar layer interfered with the disruptive effect of A23187 on the intestinal barrier. Even when we injected A23187 directly through the agar layer to stimulate the T84 monolayers, it still did not lead to the desired result. A23187 induces gut barrier disruption through the binding and transport of divalent calcium ions across the cell membrane (Fernández-Lainez et al., 2022). The drop in TEER induced by the agar layer is probably too strong to induce and measure reliably the prevention of barrier disruption. This may be due to the compression of the T84 epithelial cells by the agar blocks. Agar forms stronger gels than mucus even though colonic epithelium is covered by a mucus layer composed of a firm inner layer and a loose outer layer (Detwiler and

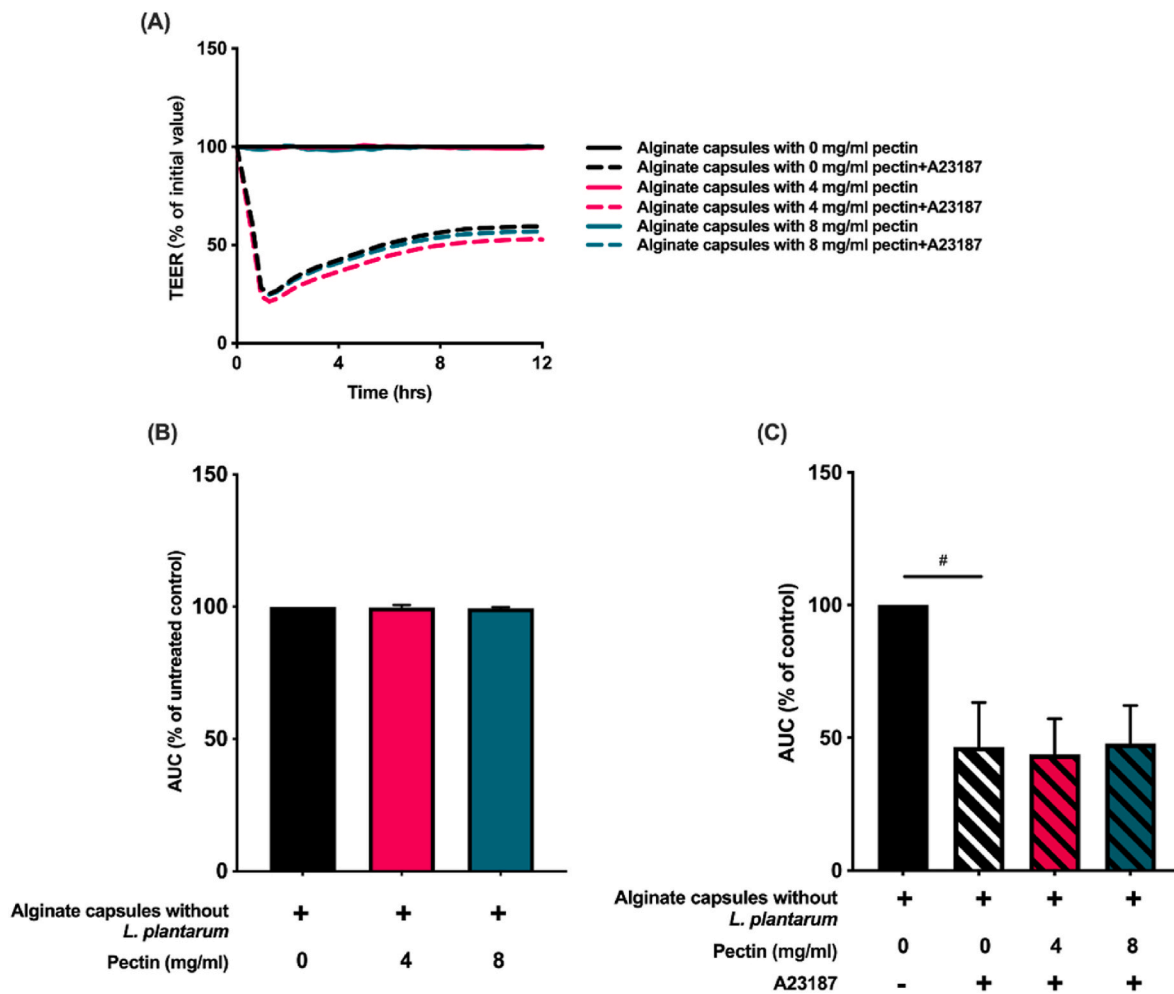


**Fig. 5.** Impact of the calcium ionophore A23187 on T84 cells in the absence and presence of alginate-encapsulated *L. plantarum* WCFS1. T84 cells were cultured for 12 h before the calcium ionophore A23187 (4  $\mu$ M) was added, and the cells were incubated for another 12 h. (A). A representative example of TEER measurement with ECIS after the addition of the disruptor A23187 in T84 cells cocultured with encapsulated *L. plantarum* WCFS1. (B). The calculated area under the curve (AUC). The AUC of untreated control was set as 100%. The % AUC of every experimental condition was calculated based on the untreated control. Statistical differences between different groups with the addition of A23187 treatment were compared with those without A23187. Data from nine independent experiments are presented as means  $\pm$  SEM. #### or \*\*\*\* $p$  < 0.0001.

Kramer, 2022; Fang et al., 2021). However, there is still debate about whether a firm mucus layer exists in the colon. Especially in the proximal colon, the mucus does not even separate the bacteria from the epithelial cells (Kamphuis et al., 2017). Therefore, we removed the agar layer in subsequent experiments but still wished to include this in this paper as a negative result to avoid other researchers trying a similar approach.

We were able to demonstrate the rescuing effects of encapsulated *L. plantarum* in the system. We added graded loads of pectin to the alginate capsules with *L. plantarum* or without *L. plantarum* to determine whether it was the fermentation of pectin or alginate that provides protective effects on barrier disruption. In addition to pectin, also alginate has been reported to modulate intestinal barrier function (Huang et al., 2021). However as shown here, we did not find any rescuing effects of pectin in alginate capsules without *L. plantarum* on A23187-induced T84 cells, but with higher pectin concentrations in

alginate-encapsulated *L. plantarum* more rescuing effects were found. We attributed the rescuing effect mainly to the impact of the fermentation of pectin in the capsules. As pectin is known to be able to directly interact with gut epithelial cells and to promote barrier function and also can serve as a substrate for fermentation and support SCFA production (Beukema et al. 2020), we next determined SCFA production in the supernatants. We only found upregulation of early intermediates, *i.e.* lactate and acetate, in the cultures. Alginate as such is already a substrate for the bacteria (Lee and Mooney, 2012; Li et al., 2016) as we found both lactate and acetate in the absence of pectin. However, acetate was lowered after the addition of the stressor which suggests that this has an impact on the fermentation processes in *L. plantarum*, but it was elevated by the addition of higher amounts of pectin. Acetate is known to enhance gut barrier function (Parada Venegas et al., 2019) and might therefore have contributed to the higher rescuing effects on A23187-induced barrier disruption. However, we found no differences



**Fig. 6.** The effect of graded loads of pectin in alginate capsules without *L. plantarum* WCFS1 on T84 epithelial intestinal barrier disruption induced by the calcium ionophore A23187. The T84 monolayer was pre-incubated with alginate capsules with graded loads of pectin (0, 4, and 8 mg/ml per capsule) for 12 h followed by a 12-h stimulation by A23187 (4  $\mu$ M). (A) The relative TEER values change in T84 cells after the addition of the A23187 and (B) alginate-pectin capsules with gradually increasing pectin concentration in absence of the disruptor. (C) The calculated area under the curve (AUC) of T84 cells pre-incubated with the alginate capsules without loads of pectin was set as 100%. The % AUC of T84 cells pre-incubated with the alginate capsules with loads of pectin was compared with the group pre-incubated with the alginate capsules without pectin. Statistical differences between different selected experimental conditions were determined. Data from three independent experiments are presented as mean  $\pm$  SEM. #  $p < 0.05$ .

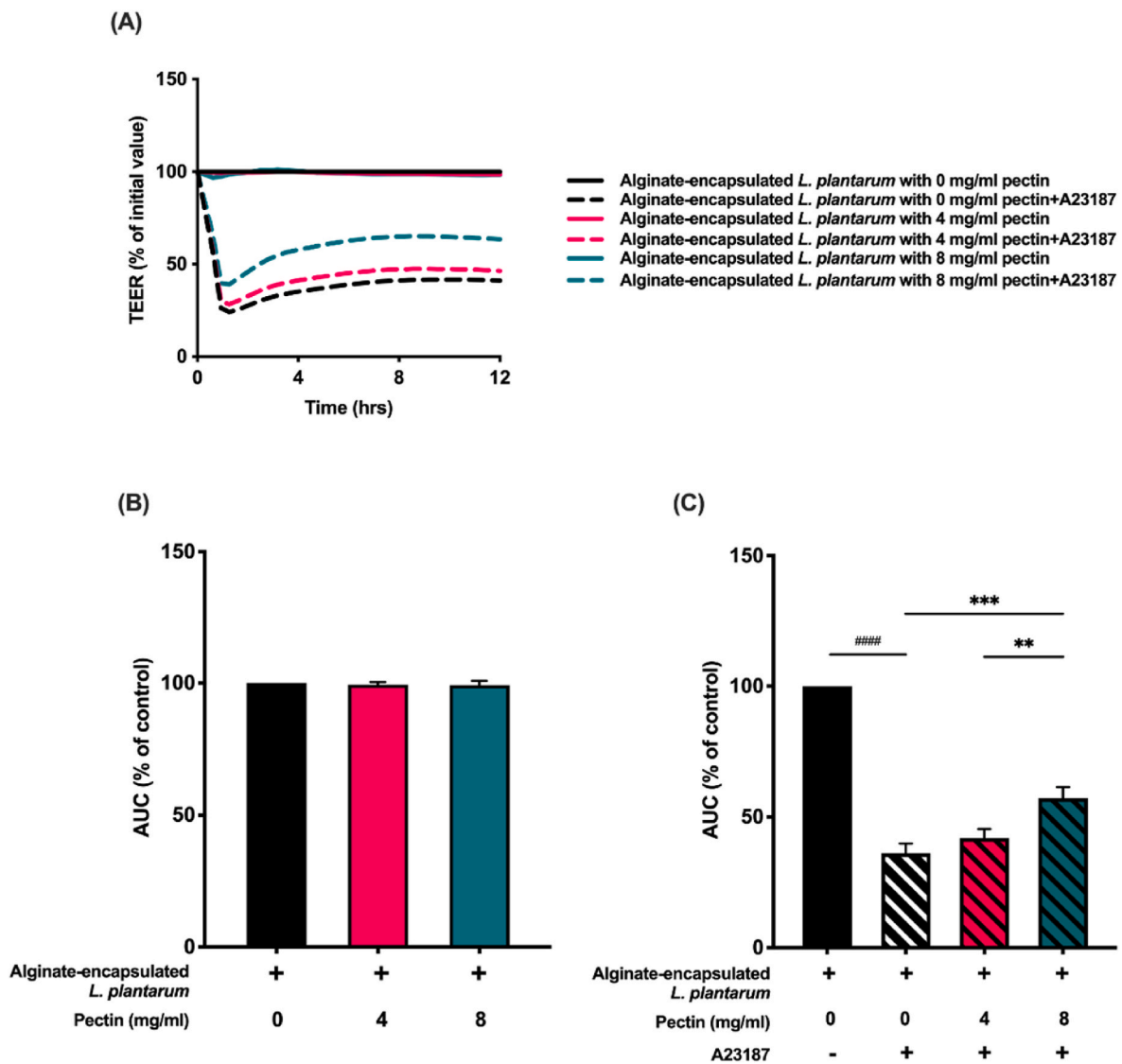
at 4 mg/ml pectin. This could imply an enhanced adsorption of the epithelial cells of acetate under A23187-induced barrier disruption as acetate might serve as fuel for the cells (Rodríguez-Enríquez et al., 2021). Although pectin in the wall of the alginate capsules without the *L. plantarum* did not have direct effects on the epithelial cells, the designed assay allows for testing both the effect of NDCs fermentation by intestinal microbiota on the intestinal epithelial barrier as well as the direct effect of NDCs on the intestinal barrier (Fig. 9).

Our data on the rescuing effects of TEER on gut epithelial cells by fermentation products corroborate the findings of others. A previous study showed that pectin's bacterial metabolites increased the TEER of Caco-2 cells in a coculture with peripheral blood mononuclear cells (Van den Abbeele et al., 2020). Also, a study using a wound healing model *in vitro* found that lactate can promote epithelial cell migration and restore the barrier to inhibit intestinal inflammation (Yu et al., 2021). Another *in vitro* experiment showed that in monolayers of T84 cells, TEER increased rapidly after administration of 40 and 80 mmol/l of acetate (around 2.4–4.8 mg/ml) and reached its highest level 30 min after administration (Suzuki et al., 2008). So, the fermentation of pectin in each group cannot be obviously seen in large differences.

Our results confirm that gut microbial metabolism exerts protecting effects on gut barrier function. Gut microbiota plays a vital role in

maintaining intestinal homeostasis, such as by regulating host metabolism and supporting proper function of the immune system (Chen and de Vos, 2023). Recently, it has also been shown that the change in intestinal bacteria might modulate the development of neurological diseases (Ullah et al., 2023). The gut microbiota utilizes undigested substances in the large intestine as substrate to produce specific microbial metabolites. The most important metabolites are SCFAs (including acetate, propionate, and butyrate) which are generated by the fermentation of NDCs (Rowland et al., 2018). These SCFAs perform many beneficial functions. They promote barrier integrity by enhancing mucus secretion and strengthening the tight junctions (Chen and de Vos, 2023). They have anti-inflammatory effects, immunomodulatory effects, and trophic effects through cellular regeneration and acceleration of healing (Gasaly et al., 2021; Kim, 2023). They reduce oxidative stress, modulate visceral sensitivity and intestinal motility (González-Bosch et al., 2021; Vissavajhala, 2017), and even prevent carcinogenesis of epithelial cells. Among them, butyrate has anticancer activity against colorectal cancer cells, inhibits oncogenesis, and activates detoxifying enzymes protecting endangered cells against toxic carcinogens (Kazmierczak-Siedlecka et al., 2022). SCFAs also have neuroprotective activities (Xiong et al., 2022). They also regulate intestinal pH influencing calcium, iron, and magnesium absorption, and have a metabolic





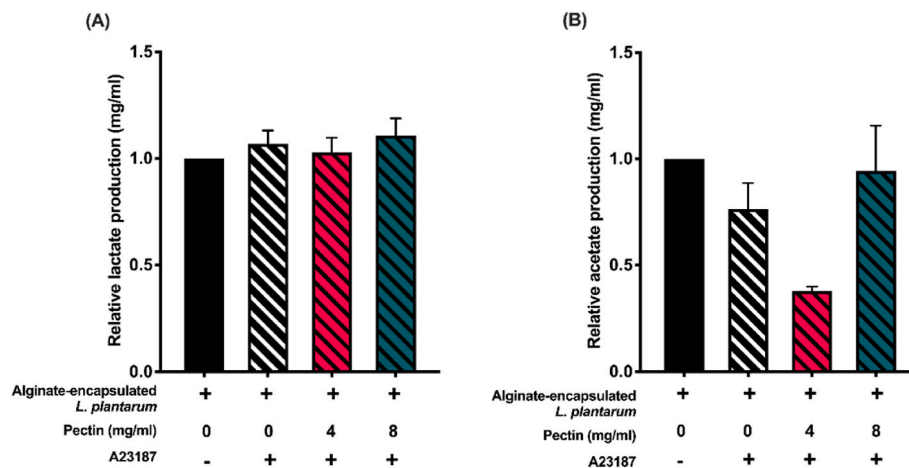
**Fig. 7.** The protective effect of graded loads of pectin in *L. plantarum WCFS1* capsules on T84 epithelial intestinal barrier disruption induced by the calcium ionophore A23187. The T84 monolayer was pre-incubated with *L. plantarum WCFS1* encapsulated by alginate with graded loads of pectin (0, 4, and 8 mg/ml per capsule) for 12 h followed by a 12-h stimulation of A23187 (4  $\mu$ M). (A). The relative TEER values change after the addition of the A23187 compared with the group that T84 cells pre-incubated with the *L. plantarum WCFS1* capsules without loads of pectin. (B) and (C). The calculated area under the curve (AUC). The AUC of T84 cells pre-incubated with the *L. plantarum WCFS1* capsules without loads of pectin was set as 100%. The % AUC of T84 cells pre-incubated with the *L. plantarum WCFS1* capsules with loads of pectin was compared with the group that T84 cells pre-incubated with the *L. plantarum WCFS1* capsules without pectin. Statistical differences between different selected experimental conditions were determined. Data from at least five independent experiments are presented as mean  $\pm$  SEM.  $^{*}p < 0.01$ ,  $^{***}p < 0.001$ , and  $^{####}p < 0.0001$ .

effect by influencing glucose metabolism (Markowiak-Kopeć and Śliżewska, 2020). However, when there is less NDC intake, the gut microbiota utilizes less beneficial energy sources, such as amino acids from exogenous or endogenous proteins or fats to produce some harmful metabolites (Peled and Livney, 2021). Our system allows for testing all these parameters and testing whether microbial metabolites have positive or negative effects.

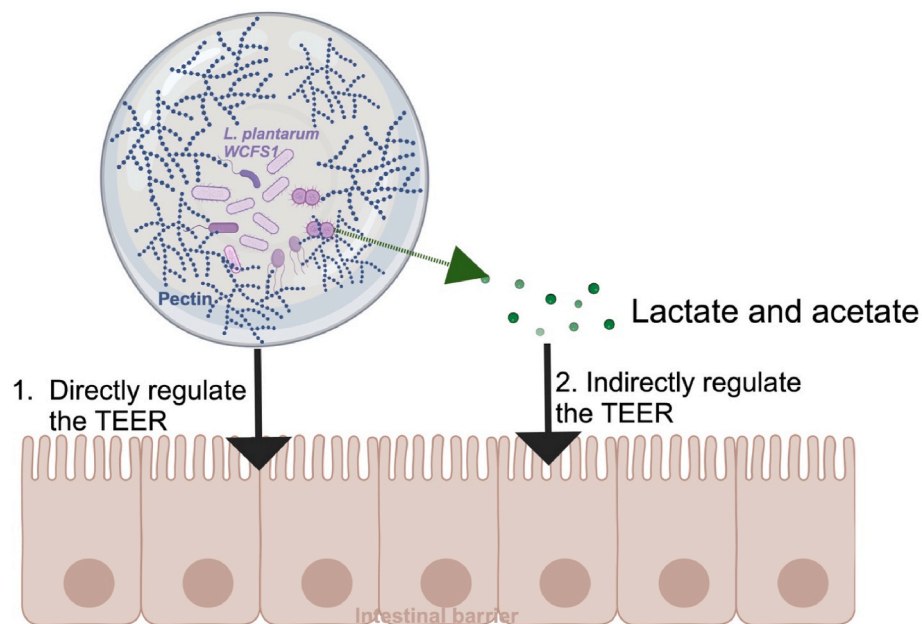
*L. plantarum* applied in our study is a kind of lactic acid bacteria (LAB) (Tannock, 2004). It is generally assumed that the supplementation of LABs in the diet has a health-promoting effect on the host (Ren et al., 2020). LABs not only ferment carbohydrates into lactic acid but also produce proteolytic enzymes providing nitrogen compounds needed for cell growth (Kieliszek et al., 2021; Waśko et al., 2012). These proteolytic enzymes enhance the digestibility of proteins and regulate the release of fatty acids (García-Cano et al., 2019). Based on the above theory, this further emphasizes the importance of bacterial fermentation

of NDC in intestinal health.

In conclusion, our study presents and tests a technology for determining the impact of real-time fermentation of NDCs by a colonic bacterium on gut barrier function. Alginate used in the capsules but also fermentable low DM high DB pectin in *L. plantarum*-containing capsules significantly enhanced the gut barrier of T84 monolayers. In future research, more types of organisms in the digestive tract can be tested in this fashion. It allows for following the production of fermentation products but also studies the direct effects of NDCs in epithelial cells and gut barrier function. Encapsulation also allows for creating an anaerobic core allowing also culturing and testing anaerobic organisms. This technology allows for a more precise follow-up of the dynamics of microbial fermentation products by bacteria on gut epithelial barrier function. This can be applied to gut bacteria but also for example to probiotic species to study in more detail their possible health effects. It is also possible to encapsulate heat-inactivated bacteria to investigate the



**Fig. 8.** The production of lactate (A) and acetate (B) produced by *L. plantarum* WCFS1 in alginate-based capsules to which graded loads of pectin was added. The capsules were co-incubated with T84 epithelial cells. The T84 monolayer was pre-incubated with *L. plantarum* WCFS1 encapsulated by alginate with graded loads of pectin (0, 4, and 8 mg/ml per capsule) for 12 h followed by a 12-h stimulation with A23187 (4  $\mu$ M). The supernatant was collected for SCFA profile analysis. Only lactate and acetate were produced. Statistical differences between different selected experimental conditions were determined. Data from at least five independent experiments are presented as mean  $\pm$  SEM.



**Fig. 9.** The designed model illustrates the involvements of two pathways to test the effects of dietary fibers such as pectin on epithelial intestinal barrier disruption induced by the calcium ionophore A23187. Pectin on the surface of the capsule may interact directly with epithelial cells to modulate the intestinal barrier or the pectin in the capsule can be fermented by *L. plantarum* WCFS1 to produce SCFAs to indirectly modulate the intestinal barrier.

possible role of bacterial factors other than fermentation metabolites. Another advantage of the current system is that it can be applied in 96-well plates and allows for a rather high-throughput manner measurement of the impact of bacteria-derived metabolites on gut barrier function.

#### CRediT authorship contribution statement

**Xiaochen Chen:** Conceptualization, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Luis Llanos Moreno:** Investigation, Validation, Writing – review & editing. **Xin Tang:** Investigation, Writing – review & editing. **Naschla Gasaly:** Investigation, Writing – review & editing. **Henk A. Schols:** Resources, Supervision, Writing – review & editing. **Paul de Vos:** Funding acquisition, Project administration, Resources, Supervision, Writing – review

& editing.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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