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Does flow culture impact upon gut-probiotic interactions: A comparison with static culture

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ABSTRACT

Conventional tissue culture models often lack physiological relevance. In this exploratory study, dynamic flow conditions were applied to 21-day cultured Caco-2 intestinal epithelial cells and their response compared with statically cultured counterparts. Host-microbiome interactions were also explored through co-incubation with Gram positive bacteria (Lab4 probiotics) or the Gram positive cell wall component, lipoteichoic acid (LTA).

Under flow, Caco-2 cells displayed increased viability, mucin production and pH tolerance compared to cells under static conditions. Measurement of secretion rates of the pro-inflammatory cytokine, interleukin-8, showed that cells under flow were unresponsive towards stimulation with Lab4 or LTA that was in complete contrast to inductions observed under static conditions. Given that Gram positive bacteria are well tolerated within the healthy gastrointestinal tract, these data suggest that flow conditions may improve the physiological relevance of Caco-2 cells. This preliminary study encourages further work exploring the impact of flow on cell-based in vitro models.

1. Introduction

The challenges associated with accessing the human gastrointestinal tract (GIT) for routine research purposes have placed considerable reliance on intestinal epithelial models in vitro (Steinway, Saleh, Koo, Delacour, & Kim, 2020). To facilitate the assessment of intestinal processes and the involvement of the gut microbiota, the most commonly used approach involves a static mono-culture of intestinal epithelium including an apical brush border and uniform tight junctions (Sambuy et al., 2005). Caco-2 cells lack adequate capability of mucin production in the monolayer format (Lea, 2015) and are often co-cultured with mucin producing HT29-MTX cells in order to improve physiological relevance (Ferraretto et al., 2018). Nevertheless, a weakness of such in vitro models is they do not reflect key physiologic aspects of the intestine; more recent developments have incorporated 3-D architecture (villi), peristalsis and dynamic flow in highly advanced and intricate systems (Rahman et al., 2021). It has been estimated that flow rates through the intestine can reach 2L per day (Cremer et al., 2016) and the application of a flow conditions to Caco-2 cell monolayers provides more meaningful biological outcomes (Rahman et al., 2021).

The flow system (Fig. 1A) is intended for use as a simple, affordable and readily available means for researchers, particularly those unfamiliar with dynamic models, to achieve physiological flow conditions in vitro and has already been shown to improve the barrier function in Caco-2 cells when compared to traditional static culture conditions (Giusti et al., 2014). Other aspects of the impact of flow on Caco-2 cell biology, such as mucin production and immuno-reactivity, are yet to be explored in the flow system. In the gastrointestinal research field there has been heavy dependence on static intestinal epithelial monolayers for probiotic screening (Raheem, Liang, Zhang, & Cui, 2021) and expectations based on capabilities in vitro do not always translate to humans.

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Cunningham et al., 2021; Kleerebezem et al., 2019) as demonstrated by the discrepancy between the probiotic induced elevation of interleukin (IL)-8 observed in vitro (Bailey, Probert, & Cogan, 2011; Malago, Tooten, & Koninkx, 2010; Tuo et al., 2018), but not observed in vivo (Walana et al., 2018). Animal and human studies indicate that the Lab4 probiotic consortium has a history of tolerability (Allen et al., 2013; Garaiova et al., 2015; Garaiova et al., 2021; Pugh et al., 2019; Tazzyman et al., 2015; Webberley et al., 2021) and has been shown to promote anti-inflammatory activities in the human gastrointestinal tract (Williams et al., 2009).

In this exploratory study, we compared the viability, mucin producing capabilities and pH tolerance of 21-day cultured Caco-2 cells exposed to standard static culture conditions and compared the outcomes with cells grown under dynamic flow conditions. Furthermore, we investigated the ability of Caco-2 flow culture conditions to impact epithelial/microbe interactions by measuring IL-8 secretion rates of cells co-incubated with the Lab4 probiotic consortium.

2. Methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (Poole, U.K.) unless otherwise stated.

2.2. Intestinal epithelial cell culture

Intestinal epithelial cell (IEC) lines were purchased from the American Type Culture Collection (ATCC, Virginia, USA). Caco-2 cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) with high glucose (4.5 g/L) supplemented with 1% (v/v) non-essential amino acids (NEAA), 10% (v/v) heat inactivated foetal bovine serum (Labtech, Sussex, UK), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C in 5% CO2 and 95% humidity. For experimentation, Caco-2 cells were seeded at 1.5x10^4 cells/cm^2 into wells of a 24 well plate (Costar, Cambridge, UK) housing a sterile 13 mm glass disc (Pyramid Innovations, Polegate, UK) and maintained for 21 days post-confluence that has been shown to facilitate cellular polarisation (Cerquetti, Serafino, Sebastianelli, & Mastrantonio, 2002; Michael et al., 2016; Natoli, Leoni, D’Agnano, Zucco, & Pelsani, 2012). HT29-MTX and HCT116 cells were cultured in a 1:1 mix of DMEM (high glucose) with McCoy’s5A (high glucose) or DMEM (high glucose), respectively, supplemented as previously described at 37 °C in 5% CO2 and 95% humidity and were used for experimentation once fully confluent (typically after 3 to 4 days of culture).

2.3. Experimental culture conditions

IEC grown on 13 mm glass discs were transferred to three different culture systems: (i) a Quasi-vivo QV500 system (Kirkstall Ltd, York, UK) with 3 inter-connected flow modules (1 disc per module) containing 15 ml of media flowing at 1.1 ml/minute (termed ‘Dynamic Flow (FLOW)’). Flow rate capabilities of the system are shown in Supplementary Fig. S1; (ii) a 30 mm diameter sterile petri dish (3 discs per dish) containing 15 ml static medium (termed the ‘Static control (STATIC)’) and providing a cell number and media volume matched control for FLOW); (iii) a well of a standard 24 well plate (1 disc per well) containing 1 ml static medium (termed ‘Conventional static control (CONV)’ and representing typical experimental conditions). A schematic of the experimental models is shown in Fig. 1B.

2.4. Cell viability

IEC viability was assessed using a 3-(4, 5-Dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as previously described (Michael et al., 2019). Viability was expressed as fold-change compared to CONV that were arbitrarily assigned as 1.

2.5. Cell surface glycoprotein

IEC were incubated with 500 μl of 1% w/v alcian blue solution (pH 2.5) for 30 min at room temperature (RT). The cells were washed 5 times with 0.05% (w/v) Tween-20 (in phosphate buffered saline (PBS)) before incubation with 500 μl of 10% (v/v) acetic acid solution for 30 min at RT. Each well was measured for absorbance at 580 nm using a spectrophotometer (Tecan). Glycoprotein levels were expressed as fold-change compared to CONV that were arbitrarily assigned as 1 and used as a surrogate marker of mucin production.
2.6. Cell cytotoxicity

IEC supernatants were centrifuged at 5000g for 5 min and the resultant cell-free supernatant was assessed for lactate dehydrogenase using the LDH-Glo™ Cytotoxicity assay (Promega, Southampton, UK) in accordance with the manufacturer’s instructions. Cytotoxicity was expressed as fold-change compared to 24-hour time points that were arbitrarily assigned as 1.

2.7. IL-8 production

IEC supernatants were centrifuged at 5000g for 5 min and the resultant cell-free supernatant was assayed for IL-8 concentration using human IL-8 ELISA kit (Peprotech, London, UK) in accordance with the manufacturer’s instructions and using an Infinite-Pro-multiplate reader (Tecan, Mannedorf, Switzerland). IL-8 level was expressed as fold-change compared to STATIC unstimulated cells (UC) that were arbitrarily assigned as 1.

2.8. IEC stimulations

Acidic pH. The pH of supplemented DMEM (serum-free) was adjusted from 7.4 to 6.5 with the dropwise addition of 1 M acetic acid for application to IEC.

Lipoteichoic acid (LTA). Lyophilized LTA (from Bacillus subtilis Sigma-Aldrich (cat no. L3265)) was reconstituted in dH2O to a final concentration of 10 mg/ml and further diluted to 10 ng/ml in supplemented DMEM (serum free) for incubation with the IEC.

Viable preparations of Lab4. De Man Rogosa and Sharpe (MRS, Oxoid, Hampshire, UK) broth (10 ml) was inoculated with freeze dried powder (10 mg) of Lab4 (composed of Lactobacillus acidophilus CUL21 (NCIMB 30156), Lactobacillus acidophilus CUL60 (NCIMB 30157), Bifidobacterium bifidum CUL20 (NCIMB 30153) and Bifidobacterium animalis subsp. lactis CUL34 (NCIMB 30172)) and incubated anaerobically (10% carbon dioxide, 10% hydrogen and 80% nitrogen) without shaking at 37 °C for 18 h. Lab4 cultures were centrifuged (1,000g, 10 min) and the resultant pellet washed with antibiotic and serum-free DMEM with 1% (v/v) NEAA and adjusted to 1x10^5 cfu/ml for application to IEC.

2.9. Statistical analysis

All data were tested for normality using the Shapiro-Wilk test and visual inspection of Q-Q plots prior to statistical analysis. Comparisons between multiple groups with normally distributed data were performed using one-way ANOVA with Holm-Šidák’s analysis post-hoc, and where the data was not normally distributed, using the Kruskal-Wallis test with Dunn’s analysis post hoc. All statistics were performed using GraphPad PRISM (Version 9.0.2, California, USA) and p values < 0.05 were considered statistically significant.
3. Results

3.1. The impact of flow on cell viability

The viability of the FLOW Caco-2 cells (Fig. 2A) was higher than that of the cell number and media volume matched static control (STATIC) and the conventional static control (CONV) at 48 and 72 h; there was a statistically significant increase in viability for FLOW compared to CONV at 48 h. The viability of other IEC lines, namely HT29-MTX and HCT116, was unaffected when incubated in conditions comparable to the Caco-2 FLOW cells after 48 h (Supplementary Fig. S2).

3.2. The impact of flow on cell surface glycoprotein production

Cell surface glycoprotein (a surrogate marker of mucin) levels were assessed after 48 h (Fig. 2B, time-matched to the significant improvements observed in Fig. 2A) and were significantly higher in FLOW when compared to STATIC (1.23-fold higher, \( p = 0.0204 \)) and CONV (2.17-fold higher, \( p < 0.0001 \)). Significantly higher levels of cell surface mucous glycoprotein were present in STATIC compared to CONV (1.77-fold higher, \( p = 0.0002 \)). FLOW did not increase mucin levels in other IEC after 48 h (Supplementary Fig. S3).

3.3. The impact of flow on pH tolerance

Lactate dehydrogenase (LDH) release (a measure of cytotoxicity (Decker & Lohmann-Matthes, 1988)) by Caco-2 cells exposed to pH 6.5 (representing the acidity of the intestinal tract (Koziolek et al., 2015)) in the FLOW and STATIC populations was assessed over the course of 48 h (Fig. 2C). For the FLOW Caco-2 cells there were no changes in LDH levels whereas in the STATIC population there was a significant increase in cytotoxicity (LDH release) between 24 h and 48 h (2.3-fold increase, \( p < 0.0001 \)).

3.4. The impact of flow on the IL-8 response to Gram-positive probiotic bacteria

As flow increased mucin production and improved pH tolerance, we next sought to establish whether these properties had an impact on the response of Caco-2 cells when incubated with bacteria. In unstimulated cells (UC) the secretion of IL-8 (a cytokine associated with gastrointestinal inflammation (Mazzucchelli et al., 1994; Okada et al., 2020)) was comparable between STATIC and FLOW (Fig. 2D). In the presence of either the Gram-positive cell wall component, lipoteichoic acid (LTA), or viable Gram-positive probiotic bacteria (the Lab4 consortium), IL-8 secretion was significantly higher for STATIC compared to FLOW (\( p < 0.0001 \) for both stimuli); significant inductions were observed in STATIC (4.2-fold, \( p < 0.0001 \) for LTA and 10.3-fold, \( p < 0.0001 \) for Lab4) whereas there was a complete lack of response for the FLOW cells. There was no reduction in viability of the Caco-2 cells despite exposure to the viable Lab4 bacteria over the duration of the experiment (data not shown).

4. Discussion

In this exploratory study we show that the application of the element of flow to confluent Caco-2 cell monolayers in vitro has no detrimental effects on cellular viability and can significantly increase cell surface glycoprotein levels and pH tolerance compared to statically cultured counterparts and may, therefore, represent a shift towards better physiological relevance. In addition, we demonstrate that Caco-2 cells exposed to flow display immuno-tolerance following exposure to both Lab4 probiotic bacteria and lipoteichoic acid. These findings support the tolerability and anti-inflammatory effects observed in Lab4 human intervention studies (Allen et al., 2013; Garaiova et al., 2015; Garaiova et al., 2021; Pugh et al., 2019; Tazzyman et al., 2015; Williams et al., 2009).

Using the flow chambers, we exposed IEC to a flow rate of 1.1 mL/min that equates to \(~1.6\) L/day at a velocity of \(~3\) μm/s at the IEC surface (calculated on the basis of modelling experiments performed by Mazzei and colleagues (Mazzei, Guzzardi, Giusti, & Ahluwalia, 2010)). It has been estimated that approximately 1.5 to 2 L of luminal fluid pass through the intestine per day (Maxwell, Kleeman, & Narins, 1994) and velocities reach 5 to 30 μm/s in the colon (Cremers, Arnoldini, & Hwa, 2017; Cremer et al., 2016).

The intestinal epithelium is covered in a complex mucin layer composed of mucous glycoproteins that function as a lubricating, protective barrier against toxic agents/pathogens and as a habitat for the intestinal microbiota (Paone & Cani, 2020). Caco-2 cells in culture are thought to produce a reduced mucin layer compared to the situation in vivo (Lea, 2015) and we have demonstrated that flow can increase cell surface mucous glycoprotein expression in these cells, indicating a potential shift towards a more physiological phenotype. These findings are consistent, at least in part, with other studies demonstrating enhanced mucin production by Caco-2 cells exposed to flow conditions using organ-on-a-chip systems (Fois, Schindeler, Valtechev, & Dehghani, 2021; Kim & Inge, 2015).

The endogenous intestinal mucin layer is dominated by MUC2 (Paone & Cani, 2020); yet expression levels of the MUC2 gene have been shown to be very low in Caco-2 cells (Grondin, Kwon, Far, Haq, & Khan, 2020). Attempts were made to measure mucin gene expression levels in our study but mRNA transcripts for MUC2, MUC5AC or MUC5B, could not be detected. Fois and colleagues also failed to detect MUC2 mRNA transcripts during flow-enhanced mucin production (Fois et al., 2021). This may indicate the involvement of other mucin types and we have found increased expression of the MUC5AC and MUC5B genes, but not MUC2, in HT29-MTX intestinal epithelium cells exposed to flow (Supplementary Fig. S4) but flow did not increase cell surface mucous glycoprotein expression by these cells.

In addition to flow, the intestinal epithelium can be exposed to acidic conditions with pH values of 5 recorded in the small intestine and colon (Koziolek et al., 2015). We observed cytotoxicity in statically cultured Caco-2 cells exposed to pH 6.5 (representing colonic pH in healthy adults (Koziolek et al., 2015)) for 48 h whereas no cytotoxicity was observed under flow conditions. These differences were possibly due to the development of a protective mucin layer that was also observed at 48 h that might be expected to afford better protection to adverse pH levels. From a methodological perspective, reductions in pH are an unavoidable consequence of static mammalian cell culture (despite the inclusion of buffering systems) and can have a profound effect on the cell behaviour that is seldom considered by researchers during data interpretation/reporting (Michl, Park, & Swietach, 2019). pH changes are amplified in co-culture experiments with probiotic bacteria that are usually acid producers. We propose that the application of flow could be particularly useful in this scenario.

Caco-2 cells are often used to screen probiotic bacteria for immunomodulatory capability (Claes et al., 2012; Malago et al., 2010; Ren et al., 2013; Roselli, Finamore, Britti, & Mengheri, 2006; Takafumi & Takaaki, 2013; Tuo et al., 2018) owing to the expression of toll-like receptors (TLRs) by the Cacos that sense bacterial antigens (Furrie et al., 2005). The expression of the pro-inflammatory cytokine IL-8 is positively associated with the pathogenesis of inflammatory bowel disease (Mazzucchelli et al., 1994) and colitis (Okada et al., 2020). In our study, stimulation of the Caco-2 cells with the Lab4 bacterial consortium induced IL-8 expression under static conditions that was completely absent in the presence of flow which may indicate the establishment of immuno-tolerance in alignment with the tolerability (no adverse effects) and anti-inflammatory effects observed during Lab4 studies in vivo (Garaiova et al., 2015; Garaiova et al., 2021; Pugh et al., 2019; Webberley et al., 2021; Williams et al., 2009). The IL-8 induction by Lab4 could, at least in part, be mediated by lipoteichoic acid (LTA) as has been seen for Lactobacillus rhamnosus GG (Claes et al., 2012) and we observed...
the complete attenuation of LTA-induced IL-8 secretion under flow conditions. LTA is a major constituent of the healthy gut microbiota and our findings align with the tolerance of the healthy human GIT to resident Gram-positive bacteria (Weiner, da Cunha, Quintana, & Wu, 2011). Lipopolysaccharide (LPS) is another major component of the gut microbiota (present in the cell membrane of Gram-negative bacteria) and, in our study, stimulation with LPS had no impact on IL-8 secretion by Caco-2 cells under static or flow conditions (data not shown). It has been reported elsewhere that Caco-2 cells are unresponsive to LPS (Abreu et al., 2001; Bocker et al., 2003; I. et al., 2003; Schuwer-Maly, Eckmann, Kagnoff, Falco, & Malay, 1994).

We are aware of a number of limitations with our work: (i) Caco-2 cell polarisation was not confirmed, (ii) only one flow rate was assessed, (iii) the shear force exerted on IEC in our model was $2.1 \times 10^{-5}$ Pa (calculated on the basis of modelling experiments performed by Mazzei and colleagues [Mazzei et al., 2010]) that is lower than expected in vivo (Lentle & Janssen, 2008), (iv) mucin gene transcripts were undetectable in Caco-2 cells that hindered any mechanistic insight into the flow mediated increases in cell surface mucous glycoprotein production, (v) the impact of flow on the secretion of other cytokines related to gastrointestinal inflammation such as IL-6 were not assessed and (vi) the single compartment (apical) design of this particular system did not allow the inclusion of experimental replicates and mid-experiment access to analyte and tissue.

Table 1

<table>
<thead>
<tr>
<th>Benefits</th>
<th>Limitations</th>
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<tbody>
<tr>
<td>Physiological relevance: capable of generating intestinal flow rates</td>
<td>Apical compartment only: not suitable for trans epithelial electrical resistance (TEER) and absorption studies</td>
</tr>
<tr>
<td>Readily available: ‘off-the-shelf’ system not requiring bespoke apparatus/equipment</td>
<td>Univariate capability: limited to the application of laminar flow conditions and lacks other physiological features such as peristaltic motion</td>
</tr>
<tr>
<td>Ease of use: intuitive protocol requiring little training</td>
<td>Cost effective: durable components can be sterilised for reuse</td>
</tr>
<tr>
<td>Modular design: allows the inclusion of experimental replicates and mid-experiment access to analyte and tissue.</td>
<td>Low-throughput</td>
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relationships which may be considered as potential competing interests: TSW, JKS, DRM, and SFP are employees of Cultech Ltd. JRM and LP are/were involved in other collaborative projects with Cultech Ltd. JRM has been paid for consultancy by Enterobiotix Ltd.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105519.

References


