Journal of Functional Foods 104 (2023) 105519

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Short communications

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

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ARTICLE INFO

Keywords:
Caco-2
Flow
Mucin
Interleukin-8
Probiotic

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 cell lines (Rahman et al., 2021) such as Caco-2 cells which, despite their malignant origin, are considered particularly useful as they spontaneously polarise during culture to display key features of the endogenous 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. 1**A**) 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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https://doi.org/10.1016/j.jff.2023.105519
Received 20 December 2022; Received in revised form 14 March 2023; Accepted 19 March 2023
Available online 27 March 2023

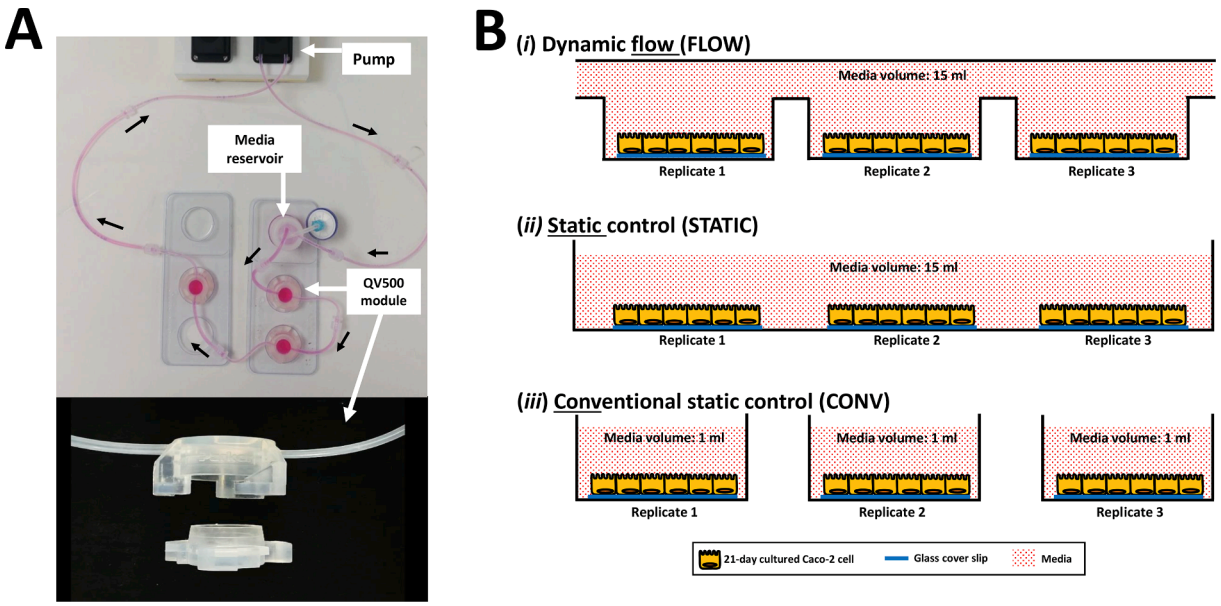

Fig. 1. (A) The Quasi-vivo flow system with 3 sequential QV500 modules. Black arrows indicate flow direction. (B) Schematic representations of experimental conditions.

(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% CO_2 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, & Felsani, 2012). HT29-MTX and HCT116 cells were cultured in a 1:1mix of DMEM (high glucose) with McCoy's5A (high glucose) or DMEM (high glucose), respectively, supplemented as previously described at 37 °C in 5% CO_2 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. 1**B**.

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.

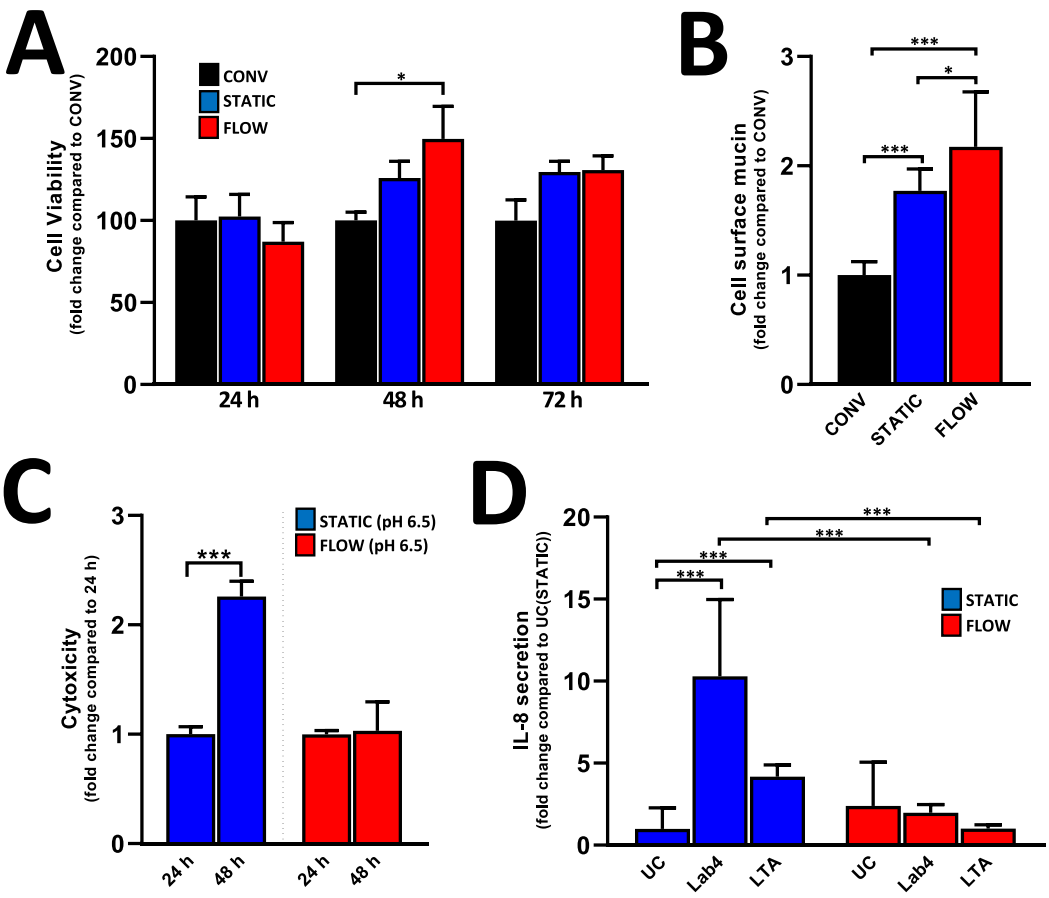

Fig. 2. The impact of flow on 21-day cultured Caco-2 enterocytes. (A) Cell viability, (B) cell surface mucous glycoprotein production, (C) pH tolerance or (D) IL-8 secretion in the absence (unstimulated control, UC) or presence of Lab4 bacteria ($1x10^5$ cfu/ml) or LTA (10 ng/ml) were assessed in 21-day cultured Caco-2 cells exposed to CONV, STATIC and FLOW conditions for up to 72 h (A) or STATIC and FLOW conditions for up to 48 h (B, C & D). Data is presented as the mean ± standard deviation (SD) of at least 3 independent experiments (with three replicate samples) and is expressed as fold-change compared to CONV (A & B), 24 h time point (C) or UC (D). Statistical analyses were performed using one-way ANOVA with Holm-Šídák's analysis *post-hoc* (A, B & C) or Kruskal-Wallis test with Dunn's analysis *post hoc* (D) where $*p < 0.05$ or $***p < 0.001$.

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 dH_2O 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-Šídák's analysis *post-hoc* or, 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. 2**A**) 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. 2**B,** time-matched to the significant improvements observed in Fig. 2**A**) 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. 2**C**). 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. 2**D**). 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 (Cremer, 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, Valtchev, & Dehghani, 2021; Kim & Ingber, 2013).

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 probotic 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 lipoteichioc acid (LTA) as has been seen for *Lactobacillus rhamnosus* GG (Claes et al., 2012) and we observed

Table 1
General considerations for the use of QV500 as a flow model of the intestinal epithelium.

Benefits	Limitations
• Physiological relevance: capable of generating intestinal flow rates • Readily available: 'off-the-shelf' system not requiring bespoke apparatus/equipment • Ease of use: intuitive protocol requiring little training • Cost effective: durable components can be sterilised for reuse • Modular design: allows the inclusion of experimental replicates and mid-experiment access to analyte and tissue.	• Apical compartment only: not suitable for trans epithelial electrical resistance (TEER) and absorption studies • Univariate capability: limited to the application of laminar flow conditions and lacks other physiological features such as peristaltic motion • Low-throughput

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 upon 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; L et al., 2003; Schuerer-Maly, Eckmann, Kagnoff, Falco, & Maly, 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 2.1×10^{-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 support assessments of barrier function or absorption capabilities for comparisons with alternative models. The clear strengths of our study are (*i*) the use of an appropriate volume and cell adjusted control (STATIC) and (*ii*) the use of 'off-the-shelf', affordable and reusable apparatus. General considerations of the QV500 system used for this study are presented in Table 1.

In summary, we propose that the application of flow conditions to cultured Caco-2 intestinal epithelial cells should be considered to help improve their biological relevance over standard static culture conditions. This may be of importance with regard to the immuno-tolerance of Gram-positive bacteria that is a pre-requisite *in vivo* for probiotic efficacy and, indeed, a non-inflammed gut. The preliminary/exploratory nature of our findings highlights the need for further work exploring the impact of flow on Caco-2 cell function, mucin production and immune responsiveness in this model and leading to studies in more advanced models with better translational relevance.

Funding.

The work was conducted as part of a Knowledge Economy Skills Scholarship (KESS2) with support, in part, from Cultech Ltd. The Division of Digestive Diseases and JRM at Imperial College London receives financial and infrastructure support from the NIHR Imperial Biomedical Research Centre (BRC) based at Imperial College Healthcare NHS Trust and Imperial College London.

Declaration of Competing Interest

The authors declare the following financial interests/personal 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

Abreu, M. T., Vora, P., Faure, E., Thomas, L. S., Arnold, E. T., & Arditi, M. (2001). Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *Journal of Immunology, 167*(3), 1609–1616. https://doi.org/10.4049/jimmunol.167.3.1609

Allen, S. J., Wareham, K., Wang, D., Bradley, C., Hutchings, H., Harris, W., … Mack, D. (2013). Lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea and Clostridium difficile diarrhoea in older inpatients (PLACIDE): A randomised, double-blind, placebo-controlled, multicentre trial. *Lancet, 382*(9900), 1249–1257. https://doi.org/10.1016/S0140-6736(13)61218-0

Bailey, J. R., Probert, C. S., & Cogan, T. A. (2011). Identification and characterisation of an iron-responsive candidate probiotic. *PLoS One1, 6*(10), e26507.

Bocker, U., Yezerskyy, O., Feick, P., Manigold, T., Panja, A., Kalina, U., … Singer, M. V. (2003). Responsiveness of intestinal epithelial cell lines to lipopolysaccharide is correlated with Toll-like receptor 4 but not Toll-like receptor 2 or CD14 expression. *International Journal of Colorectal Disease, 18*(1), 25–32. https://doi.org/10.1007/s00384-002-0415-6

Cerquetti, M., Serafino, A., Sebastianelli, A., & Mastrantonio, P. (2002). Binding of Clostridium difficile to Caco-2 epithelial cell line and to extracellular matrix proteins. *FEMS Immunology and Medical Microbiology, 32*(3), 211–218. https://doi.org/10.1111/j.1574-695X.2002.tb00556.x

Claes, I. J. J., Segers, M. E., Verhoeven, T. L. A., Dusselier, M., Sels, B. F., De Keersmaecker, S. C. J., … Lebeer, S. (2012). Lipoteichoic acid is an important microbe-associated molecular pattern of Lactobacillus rhamnosus GG. *Microbial Cell Factories, 11*(1), 161. https://doi.org/10.1186/1475-2859-11-161

Cremer, J., Arnoldini, M., & Hwa, T. (2017). Effect of water flow and chemical environment on microbiota growth and composition in the human colon. *Proceedings of the National Academy of Sciences of the United States of America, 114*(25), 6438–6443. https://doi.org/10.1073/pnas.1619598114

Cremer, J., Segota, I., Yang, C.-y., Arnoldini, M., Sauls, J. T., Zhang, Z., . . . Hwa, T. (2016). Effect of flow and peristaltic mixing on bacterial growth in a gut-like channel. *113*(41), 11414–11419. doi: doi:10.1073/pnas.1601306113.

Cunningham, M., Azcarate-Peril, M. A., Barnard, A., Benoit, V., Grimaldi, R., Guyonnet, D., … Gibson, G. R. (2021). Shaping the future of probiotics and prebiotics. *Trends in Microbiology, 29*(8), 667–685. https://doi.org/10.1016/j.tim.2021.01.003

Decker, T., & Lohmann-Matthes, M. L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods, 115*(1), 61–69. https://doi.org/10.1016/0022-1759(88)90310-9

Ferraretto, A., Bottani, M., De Luca, P., Cornaghi, L., Arnaboldi, F., Maggioni, M., … Donetti, E. (2018). Morphofunctional properties of a differentiated Caco2/HT-29 co-culture as an in vitro model of human intestinal epithelium. *Bioscience Reports, 38*(2). https://doi.org/10.1042/bsr20171497

Fois, C. A. M., Schindeler, A., Valtchev, P., & Dehghani, F. (2021). Dynamic flow and shear stress as key parameters for intestinal cells morphology and polarization in an organ-on-a-chip model. *Biomedical Microdevices, 23*(4), 55. https://doi.org/10.1007/s10544-021-00591-y

Furrie, E., Macfarlane, S., Thomson, G., Macfarlane, G. T., Microbiology, G. B., … Tumour, B. (2005). Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria. *Immunology, 115*(4), 565–574. https://doi.org/10.1111/j.1365-2567.2005.02200.x

Garaiova, I., Muchová, J., Nagyová, Z., Wang, D., Li, J. V., Országhová, Z., … Ďuračková, Z. (2015). Probiotics and vitamin C for the prevention of respiratory tract infections in children attending preschool: A randomised controlled pilot study. *European Journal of Clinical Nutrition, 69*(3), 373–379. https://doi.org/10.1038/ejcn.2014.174

Garaiova, I., Paduchova, Z., Nagyova, Z., Wang, D., Michael, D. R., Plummer, S. F., … Muchova, J. (2021). Probiotics with vitamin C for the prevention of upper respiratory tract symptoms in children aged 3–10 years: Randomised controlled trial. *Beneficial Microbes, 1–10*. https://doi.org/10.3920/BM2020.0185

Giusti, S., Sbrana, T., La Marca, M., Di Patria, V., Martinucci, V., Tirella, A., … Ahluwalia, A. (2014). A novel dual-flow bioreactor simulates increased fluorescein permeability in epithelial tissue barriers. *Biotechnology Journal, 9*(9), 1175–1184. https://doi.org/10.1002/biot.201400004

Grondin, J. A., Kwon, Y. H., Far, P. M., Haq, S., & Khan, W. I. (2020). Mucins in intestinal mucosal defense and inflammation: Learning from clinical and experimental studies. *Frontiers in Immunology, 11*, 2054. https://doi.org/10.3389/fimmu.2020.02054

Kim, H. J., & Ingber, D. E. (2013). Gut-on-a-Chip microenvironment induces human intestinal cells to undergo villus differentiation. *Integrative Biology, 5*(9), 1130–1140. https://doi.org/10.1039/c3ib40126j %J Integrative Biology

Kleerebezem, M., Binda, S., Bron, P. A., Gross, G., Hill, C., van Hylckama Vlieg, J. E., … Ouwehand, A. C. (2019). Understanding mode of action can drive the translational pipeline towards more reliable health benefits for probiotics. *Current Opinion in Biotechnology, 56*, 55–60. https://doi.org/10.1016/j.copbio.2018.09.007

Koziolek, M., Grimm, M., Becker, D., Iordanov, V., Zou, H., Shimizu, J., … Weitschies, W. (2015). Investigation of pH and Temperature Profiles in the GI Tract of Fasted Human Subjects Using the Intellicap((R)) System. *Journal of Pharmaceutical Sciences, 104*(9), 2855–2863. https://doi.org/10.1002/jps.24274

L, W., Z, D., A, O., J, S., M, J. K., & T, W. (2003). Biological activity of desulfovibrio desulfuricans lipopolysaccharides evaluated via interleukin-8 secretion by caco-2 cells. *Scandinavian Journal of Gastroenterology, 38*(1), 73–79. https://doi.org/10.1080/00365520310000474

Lea, T. (2015). Caco-2 Cell Line. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The impact of food bioactives on health: In vitro and ex vivo models* (pp. 103–111). Cham: Springer International Publishing.

Lentle, R. G., & Janssen, P. W. (2008). Physical characteristics of digesta and their influence on flow and mixing in the mammalian intestine: A review. *Journal of Comparative Physiology. B, 178*(6), 673–690. https://doi.org/10.1007/s00360-008-0264-x

Malago, J. J., Tooten, P. C., & Koninkx, J. F. (2010). Anti-inflammatory properties of probiotic bacteria on Salmonella-induced IL-8 synthesis in enterocyte-like Caco-2 cells. *Beneficial Microbes, 1*(2), 121–130. https://doi.org/10.3920/bm2009.0021

Maxwell, M. H., Kleeman, C. R., & Narins, R. G. (1994). *Maxwell & Kleeman's clinical disorders of fluid and electrolyte metabolism* (5th ed.). New York: McGraw-Hill, Health Professions Division.

Mazzei, D., Guzzardi, M. A., Giusti, S., & Ahluwalia, A. (2010). A low shear stress modular bioreactor for connected cell culture under high flow rates. *Biotechnology and Bioengineering, 106*(1), 127–137. https://doi.org/10.1002/bit.22671

Mazzucchelli, L., Hauser, C., Zgraggen, K., Wagner, H., Hess, M., Laissue, J. A., & Mueller, C. (1994). Expression of interleukin-8 gene in inflammatory bowel disease is related to the histological grade of active inflammation. *The American Journal of Pathology, 144*(5), 997–1007.

Michael, D. R., Davies, T. S., Loxley, K. E., Allen, M. D., Good, M. A., Hughes, T. R., & Plummer, S. F. (2019). In vitro neuroprotective activities of two distinct probiotic consortia. *Beneficial Microbes, 10*(4), 437–447. https://doi.org/10.3920/BM2018.0105

Michael, D. R., Moss, J. W. E., Calvente, D. L., Garaiova, I., Plummer, S. F., & Ramji, D. P. (2016). Lactobacillus plantarum CUL66 can impact cholesterol homeostasis in Caco-2 enterocytes. *Beneficial Microbes, 7*(3), 443–451.

Michl, J., Park, K. C., & Swietach, P. (2019). Evidence-based guidelines for controlling pH in mammalian live-cell culture systems. *Communications Biology, 2*, 144. https://doi.org/10.1038/s42003-019-0393-7

Natoli, M., Leoni, B. D., D'Agnano, I., Zucco, F., & Felsani, A. (2012). Good Caco-2 cell culture practices. *Toxicology In Vitro, 26*(8), 1243–1246. https://doi.org/10.1016/j.tiv.2012.03.009

Okada, T., Kanda, T., Ueda, N., Ikebuchi, Y., Hashiguchi, K., Nakao, K., & Isomoto, H. (2020). IL-8 and LYPD8 expression levels are associated with the inflammatory response in the colon of patients with ulcerative colitis. *Biomedical Reports, 12*(4), 193–198. https://doi.org/10.3892/br.2020.1280

Paone, P., & Cani, P. D. (2020). Mucus barrier, mucins and gut microbiota: The expected slimy partners? *Gut, 69*(12), 2232–2243. https://doi.org/10.1136/gutjnl-2020-322260

Pugh, J. N., Sparks, A. S., Doran, D. A., Fleming, S. C., Langan-Evans, C., Kirk, B., … Close, G. L. (2019). Four weeks of probiotic supplementation reduces GI symptoms during a marathon race. *European Journal of Applied Physiology, 119*(7), 1491–1501. https://doi.org/10.1007/s00421-019-04136-3

Raheem, A., Liang, L., Zhang, G., & Cui, S. (2021). Modulatory effects of probiotics during pathogenic infections with emphasis on immune regulation. *Frontiers in Immunology, 12*, Article 616713. https://doi.org/10.3389/fimmu.2021.616713

Rahman, S., Ghiboub, M., Donkers, J. M., van de Steeg, E., van Tol, E. A. F., Hakvoort, T. B. M., & de Jonge, W. J. (2021). The progress of intestinal epithelial models from cell lines to gut-on-chip. *International Journal of Molecular Sciences, 22*(24). https://doi.org/10.3390/ijms222413472

Ren, D. Y., Li, C., Qin, Y. Q., Yin, R. L., Du, S. W., Ye, F., … Jin, N. Y. (2013). Lactobacilli reduce chemokine IL-8 production in response to TNF-alpha and Salmonella challenge of Caco-2 cells. *Biomed Research International, 2013*, Article 925219. https://doi.org/10.1155/2013/925219

Roselli, M., Finamore, A., Britti, M. S., & Mengheri, E. (2006). Probiotic bacteria Bifidobacterium animalis MB5 and Lactobacillus rhamnosus GG protect intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic Escherichia coli K88. *The British Journal of Nutrition, 95*(6), 1177–1184. https://doi.org/10.1079/bjn20051681

Sambuy, Y., Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., & Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology, 21*, 1–26.

Schuerer-Maly, C. C., Eckmann, L., Kagnoff, M. F., Falco, M. T., & Maly, F. E. (1994). Colonic epithelial cell lines as a source of interleukin-8: Stimulation by inflammatory cytokines and bacterial lipopolysaccharide. *Immunology, 81*(1), 85–91.

Steinway, S. N., Saleh, J., Koo, B. K., Delacour, D., & Kim, D. H. (2020). Human Microphysiological Models of Intestinal Tissue and Gut Microbiome. *Frontiers in Bioengineering and Biotechnology, 8*, 725. https://doi.org/10.3389/fbioe.2020.00725

Takafumi, Y., & Takaaki, S. (2013). Lactobacillus brevis KB290 Enhances IL-8 Secretion by Vibrio parahaemolyticus-Infected Caco-2 Cells. *Journal of Microbiology and Biotechnology, 23*(1), 118–124. https://doi.org/10.4014/jmb.1207.07020

Tazzyman, S., Richards, N., Trueman, A. R., Evans, A. L., Grant, V. A., Garaiova, I., … Corfe, B. M. (2015). Vitamin D associates with improved quality of life in participants with irritable bowel syndrome: Outcomes from a pilot trial. *BMJ Open Gastroenterology, 2*(1), e000052.

Tuo, Y., Song, X., Song, Y., Liu, W., Tang, Y., Gao, Y., … Mu, G. (2018). Screening probiotics from Lactobacillus strains according to their abilities to inhibit pathogen adhesion and induction of pro-inflammatory cytokine IL-8. *Journal of Dairy Science, 101*(6), 4822–4829. https://doi.org/10.3168/jds.2017-13654

Walana, W., Ye, Y., Li, M., Wang, J., Wang, B., Cheng, J. W., … Li, F. (2018). IL-8 antagonist, CXCL8(3–72)K11R/G31P coupled with probiotic exhibit variably enhanced therapeutic potential in ameliorating ulcerative colitis. *Biomedicine & Pharmacotherapy, 103*, 253–261. https://doi.org/10.1016/j.biopha.2018.04.008

Webberley, T. S., Masetti, G., Baker, L. M., Dally, J., Hughes, T. R., Marchesi, J. R., . . . Michael, D. R. (2021). The Impact of Lab4 Probiotic Supplementation in a 90-Day Study in Wistar Rats. [Brief Research Report]. *8*(994). doi: 10.3389/fnut.2021.778289.

Weiner, H. L., da Cunha, A. P., Quintana, F., & Wu, H. (2011). Oral tolerance. *Immunology Reviews, 241*(1), 241–259. https://doi.org/10.1111/j.1600-065X.2011.01017.x

Williams, E. A., Stimpson, J., Wang, D., Plummer, S., Garaiova, I., Barker, M. E., & Corfe, B. M. (2009). Clinical trial: A multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study. *Alimentary Pharmacology & Therapeutics, 29*(1), 97–103. https://doi.org/10.1111/j.1365-2036.2008.03848.x