3-D Intestinal Scaffolds for Evaluating the Therapeutic Potential of Probiotics

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ABSTRACT: Biomimetic in vitro intestinal models are becoming useful tools for studying host–microbial interactions. In the past, these models have typically been limited to simple cultures on 2-D scaffolds or Transwell inserts, but it is widely understood that epithelial cells cultured in 3-D environments exhibit different phenotypes that are more reflective of native tissue, and that different microbial species will preferentially adhere to select locations along the intestinal villi. We used a synthetic 3-D tissue scaffold with villous features that could support the coculture of epithelial cell types with select bacterial populations. Our end goal was to establish microbial niches along the crypt–villus axis in order to mimic the natural microenvironment of the small intestine, which could potentially provide new insights into microbe-induced intestinal disorders, as well as enabling targeted probiotic therapies. We recreated the surface topography of the small intestine by fabricating a biodegradable and biocompatible villous scaffold using poly lactic-glycolic acid to enable the culture of Caco-2 with differentiation along the crypt–villus axis in a similar manner to native intestines. This was then used as a platform to mimic the adhesion and invasion profiles of both Salmonella and Pseudomonas, and assess the therapeutic potential of Lactobacillus and commensal Escherichia coli in a 3-D setting. We found that, in a 3-D environment, Lactobacillus is more successful at displacing pathogens, whereas Nissle is more effective at inhibiting pathogen adhesion.

KEYWORDS: 3-D scaffold, intestinal model, probiotics

1. INTRODUCTION

There is a significant medical need to better understand the interactions of small intestinal epithelial cells with intestinal pathogens, which contribute to and exacerbate a number of diseases including chronic diarrhea,1–3 gastroenteritis,4 and necrotizing enterocolitis.4 The virulent effects of intestinal pathogens are dependent on their ability to colonize and invade the intestinal mucosa, usually by adhering to and penetrating the epithelial layer. Antibiotics have typically been the first line of treatment for intestinal infections, yet with the increasing problem of antibiotic resistance in clinical practice, there has been a need to explore alternative antimicrobial therapies. A potential therapy or prophylactic against microbial pathogenesis is the use of probiotic strains of bacteria, including lactobacilli, bifidobacteria, and commensal Escherichia coli (e.g., Nissle 1917), which have been shown in a variety of animal models to confer beneficial effects to the intestinal mucosa by inhibiting pathogen colonization and invasion, and by modulating the host immune response.5–10 Biomimetic tissue models can provide a rapid and cheap alternative platform to study the interactions of probiotics with intestinal pathogens. At their simplest, these models are typically made up of 2-D confluent monolayers of epithelial cell types, such as Caco-2, HT-29, or HeLa, which are incubated with microbes for short-term monitoring of epithelial–microbe interactions. However, these models do not fully emulate what happens in vivo, particularly in regard to the physical three-dimensional space that the cells inhabit, despite it being well-known that bacterial colonization is greatly dependent on their 3-D niche.11–14 In response, some researchers have developed elegant microfluidic models that create a three-dimensional microenvironment15,16 and use flow mechanisms to allow simulation of biofilm formation17 and peristalsis.18 In addition, the NASA-developed rotating wall vessels (RWV) have enabled prolonged 3-D culture of both mammalian cell types and bacterial populations.19 This device has been optimized to produce laminar flow to enable the growth of intestinal organoids in suspension culture in conjunction with bacteria to simulate an enteric infection in a fluidic setting. However, thus far the specific three-dimensional surface topography of the intestine has been poorly recreated, i.e., via re-creation of the intestinal villi. Epithelial cells typically become more differentiated and polarized while moving along the crypt–villus axis, and they subsequently express different apical and basolateral receptors. It has been shown in vitro by previous researchers that many strains of bacteria will preferentially adhere to epithelial cells in different stages of differentiation. For instance, Salmonella, enteropathogenic E. coli, and Listeria all target receptors such as microvilli on differentiated cells residing on the villi,20–25 whereas Yersinia...
Pseudotuberculosis and Pseudomonas have been shown to preferentially adhere to unpolarized, less differentiated cells, which are found in the crypt regions of real intestines. Salmonella has been shown to interact with the microvilli of polarized enterocytes and induce membrane ruffling through cytoskeleton reorganization, allowing them to penetrate the epithelial layer. Two of the drawbacks to traditional (2-D) models is that they rarely allow for both undifferentiated and differentiated epithelial cell types to be cultured at the same time and they completely ignore the physical dimensions typical of villus structures. We have previously demonstrated that small sections of synthetic 3-D intestine can be synthesized from collagen, silicon, and poly lactic-glycolic acid (PLGA) with realistic villus geometries, which can be used to support the growth and differentiation of epithelial cell types in a manner similar to real intestinal tissue. In this study, we aimed to show that these intestinal models can also be used to evaluate the therapeutic potential of two intestinal probiotics (Lactobacillus gasseri and E. coli Nissle 1917) against two intestinal pathogens (Salmonella typhimurium and Pseudomonas aeruginosa) in a more realistic physiological setting. Three scenarios of bacterial adhesion were tested: displacement, competition, and inhibition. Displacement refers to the ability of the probiotic to physically remove an intestinal pathogen after it has established an adhesive niche on the epithelial cells. Competition refers to the ability of the probiotic to compete with the pathogen for adhesive binding sites on the epithelial cells, assuming that the starting inoculum is the same concentration. Inhibition refers to the ability of the probiotic to establish an adhesive niche on the epithelial cells, and then retain this niche once exposed to pathogen, thereby blocking the pathogen from binding.

2. MATERIALS AND METHODS

2.1. Fabrication of Intestinal Scaffolds. Porous PLGA scaffolds with intestinal villus features were fabricated as described previously. Briefly, micromolding techniques were used to create agarose molds of 500 μm deep, high aspect ratio holes from a poly(methyl methacrylate) (PMMA) template. PLGA (100 mg/mL in chloroform, from Lactel Absorbable Polymers, Birmingham, AL) was mixed with a porogen (sodium bicarbonate, 400 mg/mL) and homogenized for 2 min. Intestinal scaffolds were formed by coating the agarose molds with the PLGA–porogen solution under vacuum, followed by freezing at -20 °C overnight and then immersion in precooled ethanol for 12 h to extract the chloroform. The scaffolds were then immersed in warm distilled water for 24 h to dissolve the porogen, and sterilized with 70% ethanol for 24 h prior to use. Prior to cell seeding, the PLGA scaffolds were placed into a custom designed scaffold-insert kit from previously reported methods, and then soaked overnight in coculture media which was added to both basolateral and apical compartments.

2.2. Cell Culture on Transwell Inserts. Caco-2 cells (ATCC, Manassas, VA) passage 18–25, were expanded and maintained in tissue culture medium [DMEM with 10% fetal bovine serum (FBS), 1× antimycotic—antibiotic, and 1% nonessential amino acids] (all from Invitrogen, Long Island, NY). Cells were maintained in a humidified 37 °C incubator with 5% CO2, with regular passage 1–2 times a week and medium change every 2 days. Caco-2 were removed from culture flasks with 0.25% (v/v) trypsin, 0.02% EDTA solution in PBS and seeded onto the Transwell insert scaffolds at a concentration of 1 × 105 cells/mL and grown for 21 days, and 1 × 107 cells/mL and cultured for 4 days to produce differentiated and undifferentiated monolayers, respectively. Medium was added to both the basolateral and apical compartments and replaced every 2 days thereafter, and antibiotics were removed from the tissue culture medium the night before bacterial seeding.

2.3. Cell Culture on PLGA Scaffolds. Caco-2 cells were maintained as in section 2.2. Cells were seeded onto the PLGA scaffolds at a concentration of 1 × 105 cells/mL. Medium was added to both the basolateral and apical compartments and replaced after a 30 min cell attachment period, and replaced every 2 days thereafter. Experiments were performed at 21 days post Caco-2 seeding to enable cell differentiation along the crypt–villus axis, and antibiotics were removed from the tissue culture medium the night before bacterial seeding.

2.4. Bacterial Strains and Growth Conditions. Salmonella typhimurium 14038 (ST), Pseudomonas aeruginosa 15692 (PAO1), and Lactobacillus gasseri 33323 (LAB) were from ATCC, Manassas, VA. Escherichia coli Nissle 1917 (Nissle) was obtained from a commercial preparation of the probiotic Mutafal as described previously. Overnight cultures of Nissle, ST, and PAO1 were grown in LB medium, and LAB were grown in Difco Lactobacilli MRS medium (all from BD, Franklin Lakes, NJ). Cultures were maintained at 37 °C, with shaking at 225 rpm.

2.5. Bacterial Adhesion Assay on Transwell Inserts. The bacterial adhesion assay was performed as previously described, but with some modifications. A preculture of bacteria was grown for 16 h, before diluting 1:50 in fresh medium and grown back to midexponential phase for a further 1.5 h. Bacteria were then adjusted to a final concentration of 5 × 107 cells/mL in an even mixture of bacterial medium and DMEM (no antibiotics), and 1 mL of bacterial suspension was then added to the apical surface of the Caco-2-covered Transwells, with incubation at 37 °C for 2 h. Nonadhered bacteria were removed by washing twice in PBS, and cells were removed from the scaffolds by incubating with 500 μL of trypsin–EDTA at 37 °C for 10 min. The reaction was blocked with 500 μL of DMEM containing FBS, and serial 10-fold dilutions were plated onto MRS agar for LAB, and MacConkey agar (EMD Millipore, Billerica, MA) for ST, PAO1, and Nissle, with incubation for 24 h at 37 °C. MacConkey agar selects for lactose-fermenting Nissle (pink colonies) against non-lactose-fermenting ST and PAO1 (yellow-brown colonies).

2.6. Bacterial Adhesion Assay on PLGA Scaffolds. The bacterial adhesion assay was performed as in section 2.5, but bacteria were seeded onto the apical surface of PLGA scaffolds in the inset kits, instead of Transwells. The following scenarios of bacterial adhesion were tested: probiotic displacing pathogen (2 h incubation with ST or PAO1 followed by 2 h incubation with Nissle or LAB, at either 1:1 or 3:1 probiotic to pathogen ratios); probiotic inhibiting pathogen adhesion (2 h incubation with Nissle or LAB followed by 2 h incubation with ST or PAO1, at either 1:1 or 3:1 probiotic to pathogen ratios); probiotic competing with pathogen (2 h incubation with mixture of Nissle or LAB and ST or PAO1, at either 1:1 or 3:1 probiotic to pathogen ratios). Adhesion was expressed as log10 CFU/mL.

2.7. Bacterial Invasion Assay. The invasive ability of ST and PAO1 was assessed using the gentamicin protection assay. Bacterial adhesion scenarios to the Caco-2 surface were set up as described in section 2.4. Nonadhered bacteria

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were removed by washing twice in PBS, followed by incubation with 1 mL of gentamicin (150 μg/mL−1 in DMEM) for 1 h at 37 °C to kill the adhered extracellular bacteria. Dead bacteria were removed by washing twice in PBS, followed by an incubation with 500 μL of 0.1% Triton X-100 for 15 min at 37 °C to lyse the Caco-2 and release the intracellular (invaded) bacteria. Serial fold dilutions and plating were then employed as described in section 2.4.

2.8. Transepithelial Electrical Resistance (TEER). To measure TEER values of the Caco-2 monolayers before and after bacterial treatments, the medium was aspirated from the insets, replaced with fresh DMEM both basolaterally and apically, and incubated at 37 °C for 15 min. TEER was measured with an EVOM2 epithelial voltohmmeter with STX3 electrodes (World Precision Instruments, Sarasota, FL). Electrodes were placed on the apical and basolateral sides of the inset kits, and the resistance was corrected for surface area (0.5 mm) and expressed as Ω·cm². The intrinsic resistance (scaffold) was subtracted from the total resistance (scaffold and Caco-2 cells ± bacteria) to give the monolayer resistance.

Figure 1. Confocal microscopy of PLGA scaffolds cultured for 21 days with Caco-2 (blue), and 2 h with bacteria (red and green). A = Nissle, B = LAB, C = ST, and D = PAO1. 20x magnification shows full coverage of scaffolds with Caco-2 and bacteria (A-D1) with a zoomed in 40x magnification enabling visualization of individual bacteria (A-D2), and 3-D rendering shows bacteria adhering selectively to different locations along the crypt–villus axis on an individual villus measuring 500 μm (A-D3).
2.9. MTT Assay. MTT assays were used to assess Caco-2 cell viability in the presence of bacteria using a vybrant MTT assay proliferation kit (Invitrogen) according to the manufacturer’s instructions with some modifications. Briefly, bacterial
adhesion scenarios to the Caco-2 surface were set up as described in section 2.4 and extracellular bacteria were removed with gentamicin as described in section 2.5. After washing twice in PBS, Caco-2 were removed from the scaffold surface by a 10 min incubation with 500 μL of trypsin–EDTA at 37 °C. Cells were centrifuged for 5 min at 150g, resuspended in DMEM, and adjusted to a cell density of 1 × 10⁵ cells/mL. A 100 μL suspension of each cell sample was added to a 96 well plate and incubated for 36 h at 37 °C with 5% CO₂. The wells were replaced with fresh medium containing 10 μL of 12 mM MTT stock solution and incubated for 4 h at 37 °C, followed by incubation with 100 μL of SDS–HCl solution for a further 4 h. The absorbance was read at 570 nm, and cell viability was assessed against control samples of intestinal scaffolds that had not been exposed to bacteria, and expressed as % cell survival.

2.10. FISH and Confocal Imaging. Fluorescence in situ hybridization (FISH) enabled visualization of the adherent bacteria on the Caco-2 monolayers, using the following probes: TTT CAT CTG GTG CAA GCA CC (LAB); TCT CGG CCT TGA AAC CCC (PAO1) AAT CAC TTC ACC TAC GTG (ST); TT-FISH-CAC CGT AGT GCC TCG TCA CCT TGA AAC CCC (PAO1) AAT CAC TTC ACC TAC GTG (ST); TT-FISH-CAC CGT AGT GCC TCG TCA CCT TGA AAC CCC (PAO1) AAT CAC TTC ACC TAC GTG (ST). Intestinal cell-coated PLGA scaffolds were fixed with 4% paraformaldehyde for 20 min at room temperature, then dehydrated by submerging in 50% ethanol for 3 min, 80% ethanol for 3 min, and then 100% ethanol for 3 min. Samples were incubated overnight in a humidified chamber at 45 °C with 5% CO₂. The majority of the Nissle (Figure 1A) and PAO1 (Figure1C) cells located to the base of the scaffold where the undifferentiated cells resided, whereas most of the LAB and ST, primarily located to the tips of the villi, on the differentiated cells. Figure 2 shows colony counts from 2-D cultures of Caco-2 (no villi), cultured to the tips of the villi, on the differentiated and differentiated states were verified by TEER as described previously. Comparison of the two data sets. For both LAB and ST, the highest number of cells adhered to the 21 day differentiated Caco-2 cultures, and for both Nissle and PAO1 the highest counts were on the 4 day undifferentiated cultures. Figure 3 shows the location of the bacteria on the intestinal scaffolds when used in a 1:1 pathogen/probiotic ratio. The images show that Nissle and ST have a different adhesive niche (Figure 3A), compared to Nissle and PAO1, which share a similar adhesive niche in the crypt region (Figure 3B). In contrast, LAB and ST are located in similar positions with the differentiated cells on the villus

Figure 4. Colony counts from a 2 h incubation of Caco-2 cultures with ST, with results showing adhesion to the Caco-2 surface, or invasion into the Caco-2 cells, expressed as log⁵ CFU/mL. The colony counts of ST in isolation were compared to colony counts of ST when treated with probiotic LAO1 (A) or Nissle (B); DP = displacement, I = inhibition of adhesion, and C = competition. Significance was assessed with an unpaired t test, followed by a Bonferroni correction post test to determine significance across the multiple scenarios (p < 0.008). The Bonferroni significances were plotted on the graphs.

2.11. Statistical Analysis. TEER, all cell counts, and MTT assays were performed in triplicate, and data are presented as means ± SD. Statistical differences were determined by using a Student’s unpaired t test, with p values of less than 0.05 being considered significant (α = 0.05). For experiments in which we had 12 scenarios of adhesion we used a Bonferroni correction and divided α by 12 and hence regarded p values of less than 0.004 as statistically significant. We also used a Bonferroni correction on plate counts; however, in this scenario, since we separated the probiotics into 2 separate experiments that looked at both adhesion and invasion, we divided α by 6.

3. RESULTS

3.1. Adhesion and Location of Bacteria on Intestinal PLGA Scaffolds. FISH and confocal microscopy was used to determine the location of the four strains of bacteria to the Caco-2 monolayers on intestinal PLGA scaffolds (Figure 1). The images show a clear difference in adhesive niche along the crypt–villus axis between the strains of bacteria. The majority of the Nissle (Figure 1A) and PAO1 (Figure1C) cells located to the base of the scaffold where the undifferentiated cells resided, whereas most of the LAB and ST primarily located to the tips of the villi, on the differentiated cells. Figure 2 shows colony counts from 2-D cultures of Caco-2 (no villi), cultured on polyester Transwell inserts for 4 days and 21 days, which produce undifferentiated and differentiated monolayers, respectively. Differentiation states were verified by TEER as described previously. Comparison of the two data sets. For both LAB and ST, the highest number of cells adhered to the 21 day differentiated Caco-2 cultures, and for both Nissle and PAO1 the highest counts were on the 4 day undifferentiated cultures. Figure 3 shows the location of the bacteria on the intestinal scaffolds when used in a 1:1 pathogen/probiotic ratio. The images show that Nissle and ST have a different adhesive niche (Figure 3A), compared to Nissle and PAO1, which share a similar adhesive niche in the crypt region (Figure 3B). In contrast, LAB and ST are located in similar positions with the differentiated cells on the villus...
whereas the majority of LAB and PAO1 were in different positions.

3.2. Protective Effects of Probiotics against Adhesion and Invasion of ST. The adhesive and invasive activity of ST and the potential therapeutic effects of the probiotics were assessed through colony counts for adhesion and invasion, measurements of tight junction integrity through TEER, and Caco-2 cell survival using an MTT assay. The probiotics were assessed for their ability to displace ST from the scaffolds, inhibit the adhesion of ST, or compete with the pathogen for colonization space, as well as how these scenarios affected the invasion of ST into the Caco-2 cells. Figure 4A shows that LAB reduced the adhesion of ST to the Caco-2 through displacement, inhibition, and competition. Reduction in adhesion through displacement and competition appeared to be a dose-dependent event, with a greater reduction being obtained by using a 3:1 ratio of LAB to ST. Using the Bonferroni post test it was found that, with a 3:1 ratio of LAB to ST, a significant reduction of ST adhesion occurred via displacement. A 3:1 ratio of LAB used in the displacement assay also gave the greatest reduction in invasion into the Caco-2 monolayers. Similarly, the reduction of ST using Nissle as the probiotic appeared to be a dose-related event, and none of the 1:1 adhesion scenarios were significant. However, in contrast to LAB, which was most effective at displacing ST, Nissle was most effective against ST when used as a high-dose pretreatment to inhibit adhesion. Figure 5A shows that, in isolation, ST reduces the TEER of Caco-2 monolayers compared to a bacteria-free control. Both LAB and Nissle increased TEER values for Caco-2 cells growing on the scaffolds in a dose-dependent manner, despite the presence of ST. This increase was significant for all three scenarios of adhesion with a 3:1 ratio of probiotic to pathogen.
In addition, the percentage cell survival significantly increased with the addition of LAB and Nissle (Figure 7B). Applying a 3x probiotic treatment to the Caco-2 before the addition of ST promoted the highest level of cell survival, with no difference to control samples without ST (i.e., 100% cell survival). The displacement and competition scenarios at a 3:1 ratio enabled over 80% of the cells to survive, with no significant difference between the two. Nissle appeared to promote higher levels of cell survival compared to LAB at lower concentrations (1:1) however, with a significant difference in all three scenarios tested.

3.3. Protective Effects of Probiotics against Adhesion and Invasion of PAO1. The adhesive and invasive potential of PAO1 and the subsequent therapeutic effects of the probiotics were assessed using colony counts, TEER, and an MTT assay. It was found that LAB significantly lowered the number of adhered ST to the Caco-2 in all scenarios tested except the 1:1 inhibition assay (Figure 6A). Invasion was not significantly reduced in any case, even with higher dose of LAB. It was found that Nissle significantly lowered the number of adhered ST to the apical surface of the Caco-2 in most scenarios tested, except the 1:1 competition assay. In contrast to LAB, Nissle managed to significantly reduce invasion into the Caco-2 cells in every scenario with a high dose of probiotic (Figure 6B).

As with the pathogen ST, Figure 7A shows that, in isolation, PAO1 reduced the TEER of Caco-2 monolayers compared to a bacteria-free control, and that both probiotics raised TEER values when used in conjunction with PAO1. Again, the rise in TEER was dose-dependent: higher concentrations of probiotic had a more significant effect across all scenarios tested. Displacement and competition treatments raised the TEER more than inhibition, however, there was little difference between the two strains of probiotic. However, there was a difference in how the two probiotics affected cell survival.

Although the percentage cell survival was significantly improved in all scenarios tested, treatments with Nissle fared better than LAB in all cases except a 1:1 competition (Figure 7B).

4. DISCUSSION

Biomimetic tissue cell models can be used to simplify studies on bacterial–host cell interactions to determine the effects of one or two specific factors. In this study, we looked at the effects of intestinal epithelial cell differentiation on the adhesion and invasion of two pathogens, and the subsequent potentially therapeutic or prophylactic effects of two well-known probiotics. It has been shown extensively in the literature that the differentiation of Caco-2 is a growth related process that closely mimics the differentiation profiles of small intestinal epithelium in vivo with undifferentiated cells during exponential growth that turn into polarized and differentiated cells at postconfluence. In the small intestine, this process occurs along the crypt–villus axis, with dividing nondifferentiated cells residing near the crypt regions that move up the villi to become more differentiated cells. We have shown in our previous studies that we can mimic this phenomenon using a 3-D villus scaffold to support the directional growth and differentiation of Caco-2 nondifferentiated intestinal epithelial cells express basolateral markers over the entire surface (including cadherins, integrins, etc.) whereas differentiated epithelial cells also display apical markers, including brush border enzymes and microvilli. It has been shown in previous in vivo studies that certain strains of bacteria will preferentially adhere to epithelial cells in different stages of differentiation. The pathogen Salmonella has been shown to interact with the apical surface of differentiated epithelial cells in the intestine, which is accompanied by a degeneration of microvilli upon invasion. In contrast, some strains of Pseudomonas may preferentially adhere to and invade nondifferentiated cells. Also, through wound-healing experiments, it...
was shown that higher levels of *Pseudomonas* interact with cells that had reverted back to their nondifferentiated state.\(^{27,41}\) It has been suggested that *Pseudomonas* may preferentially interact with basolateral receptors, although it is worth noting that this phenomenon has not been as widely characterized as *Salmonella*. We have shown that, using both traditional 2-D Transwell inserts and our biomimetic model, the ecological niche of both ST and PAO1 resembles that of previously reported methods, with the majority of adhesion at differentiated cells at the tips of the villi and undifferentiated cells near the crypt region, respectively. In addition, the higher levels of LAB adhesion to the differentiated cells at the tips of the villi were in agreement with the literature.\(^{42}\) Some strains of *E. coli* have been found to interact mainly with differentiated cell types,\(^{43}\) however, in our 3-D model we found that the majority of Nissle was located near the undifferentiated cells at the base of the villi, and this could potentially be due to the variation in adhesins expressed by different strains of *E. coli* (Nissle for example has no S-fimbriae and inactive P-fimbriae\(^{44}\)). As a further demonstration of the different bacterial niches in the small intestine, we also showed that two strains of bacteria could be cultured in a 3-D model in different locations along the crypt–villus axis. Our hypothesis was that the therapeutic potential of probiotics against intestinal pathogens may be altered based on the differences in ecological niche.

In our 3-D intestinal model, we found that both probiotics tested successfully reduced the adhesion and invasion of ST into Caco-2 monolayers in a dose-dependent manner, in good agreement with the literature, which has shown extensively that probiotics were most effective at displaying pathogenic bacteria once it had colonized, and Nissle was more effective at preventing attachment. With further experimentation, we believe that this system could provide a platform for more specific targeting of probiotics to certain intestinal pathogens, for example to determine which probiotics are best to be taken routinely as an inhibitory measure, and which can be used for elimination of an infection once established.

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