

RESEARCH ARTICLE

Maturation of human intestinal organoids in vitro facilitates colonization by commensal lactobacilli by reinforcing the mucus layer

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Abstract

Lactobacilli, which are probiotic commensal bacteria that mainly reside in the human small intestine, have attracted attention for their ability to exert health-promoting effects and beneficially modulate host immunity. However, host epithelial-commensal bacterial interactions are still largely unexplored because of limited access to human small intestinal tissues. Recently, we described an in vitro maturation technique for generating adult-like, mature human intestinal organoids (hIOs) from human pluripotent stem cells (hPSCs) that closely resemble the in vivo tissue structure and cellular diversity. Here, we established an in vitro human model to study the response to colonization by commensal bacteria using luminal microinjection into mature hIOs, allowing for the direct examination of epithelial-bacterial interactions. *Lactobacillus reuteri* and *Lactobacillus plantarum* were more likely to survive and colonize when microinjected into the lumen of mature hIOs than when injected into immature hIOs, as determined by scanning electron microscopy, colony formation assay, immunofluorescence, and real-time imaging with *L. plantarum* expressing red fluorescent protein. The improved mature hIO-based host epithelium system resulted from enhanced intestinal epithelial integrity via upregulation of mucus secretion and tight junction proteins. Our study indicates that mature hIOs are a physiologically relevant in vitro model system for studying commensal microorganisms.

Abbreviations: 3D, three-dimensional; AB, alcian blue; CFU, colony-forming unit; GI, gastrointestinal; hIOs, human intestinal organoids; hPSCs, human pluripotent stem cells; hSI, human intestine; IL-2, interleukin-2; MRS, de Man, Rogosa, and Sharpe; PAS, periodic acid-Schiff; qPCR, quantitative real-time PCR; RFP, red fluorescent protein; SEM, scanning electron microscopy; spp., species; ZO-1, zonula occludens-1.

KEYWORDS

colonization, host-microbe interaction, human intestinal organoid, lactobacilli, microinjection, mucus

1 | INTRODUCTION

The epithelium of the gastrointestinal (GI) tract serves complex roles, including nutrient uptake and transport, inter-organ signaling, providing a protective barrier against external environmental agents^{1,2} and presenting an interface for host-microbe interactions.³ The GI tract contains a diverse microbiota, an interconnected community of symbiotic, commensal and, pathogenic microorganisms⁴ including bacteria, fungi, viruses, and parasites, which influence adaptive immunity and autoimmunity,⁵ nutrient and drug metabolism,⁶ cognitive and emotional responses,⁷ and disease states.⁸ Therefore, there have been growing demands for novel co-culture systems that can be used to explore the interactions between microbes and the human gut epithelium.

GI organoids facilitate methodological advancements in the field of host-microbe interactions by providing in vitro human model systems to investigate how microbes interact with, penetrate, and influence the GI epithelium.⁹ Both adult tissue-derived epithelial organoids and human pluripotent stem cell (hPSC)-derived GI organoids, which structurally and physiologically resemble human tissues, have been used to study host-microbe and host-pathogen interactions.^{10,11} These organoids have a three-dimensional (3D) open luminal structure that is lined by an epithelium containing four major cell types of the small intestine, including enterocytes, Paneth, goblet, and enteroendocrine cells, as well as stem cells. Microbes can be microinjected into the lumen of such organoids, which is closely akin to the GI lumen in vivo, enabling the study of direct interactions between the host epithelium and microbes and investigation of the pathophysiology of various microbe-associated diseases.¹²

Recently, hPSC-derived intestinal organoids (hIOs) and gastric organoids (hGOs) have become integral tools in studying infection mechanisms underlying host-pathogenic bacterial interactions,¹³ including *Salmonella*,¹⁴ *Clostridium difficile*,¹⁵ Shiga toxin producing-*Escherichia coli* O157:H7,¹⁶ and *Helicobacter pylori*.¹⁷ Though these organoids offer great advantages in modeling host-microbe interactions, their success likely depends on the similarity to their in vivo counterparts. It was previously reported that hPSC-derived organoids fail to develop into functional, mature tissues in vitro when applying conventional organoid protocols.^{11,18,19} Recently, our group demonstrated that immature hIOs mimicking the fetal intestine can rapidly mature into adult-like functional hIOs by co-culture with human T lymphocytes or treatment with interleukin-2 (IL-2).²⁰ These in

vitro-matured hIOs exhibit structural and functional features of the adult human small intestine, including mature goblet cells and a relatively thick mucus layer, which is located at the interface between most enteric bacteria and the host epithelium, and is the first line of host defense against pathogen invasion.²¹

In the present study, we demonstrate that in vitro-matured hIOs can serve as a proper research model for commensal bacteria, such as lactobacilli. For example, Lactobacilli are the most widely used probiotic agents applied to maintain intestinal barrier integrity and to treat various GI disorders.²² While most gut microorganism populations are obligate anaerobes, *Lactobacillus* species (spp.) are facultative anaerobic commensal bacteria that mainly reside in the small intestine.²³ We found that various *Lactobacillus* spp. can colonize and replicate within the lumen of immature hIOs. Furthermore, microinjection of *Lactobacillus reuteri* and *Lactobacillus plantarum* into the lumen of mature hIOs resulted in more effective bacterial colonization in vitro than microinjection into immature hIOs. We hypothesized that these phenomena were due to improvement in intestinal integrity in the mature hIOs by inducing mucus secretion and tight junction protein expression. Based on molecular and structural features, adult-like, mature hIOs may offer an excellent in vitro model system to study the interactions among the host epithelium and commensal bacteria and diseases associated with the disturbance of the commensal microbiota.

2 | MATERIALS AND METHODS

2.1 | hPSC-derived 3D hIO differentiation and maturation

Human embryonic stem cells (hESCs) (H9) were purchased from WiCell Research Institute (Madison, WI, USA). The hESCs were cocultured with gamma-irradiated mouse embryonic fibroblast (γ -MEF) in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% of serum replacement (SR, Gibco), 8 ng/mL of basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA), 1% of penicillin/streptomycin (P/S, Gibco), 1% of GlutaMAX (Gibco) and beta-mercaptoethanol (Gibco). The cells were passaged weekly using a clump dissociation method.²⁴ hPSC-derived hIOs were generated as previously reported.^{25,26} hESCs were dissociated and seeded in mTeSR1 basal

medium (Stemcell Technologies, Vancouver, Canada) in a culture plate coated with Matrigel (Corning, Corning, NY, USA). When the hPSCs reached 80%-90% confluency, the medium was replaced with definitive endoderm induction medium containing 100 ng/mL of Activin A (R&D Systems) and 0%-2% of fetal bovine serum (FBS, Gibco). After 3 days, the cells were incubated in hindgut differentiation medium containing 3 μ M CHIR99021 (Torcris, Ballwin, MO, USA) and 250 ng/mL of fibroblast growth factor 4 (FGF4, PeproTech, Rocky Hill, NJ, USA) for 4-6 days. The hindgut spheroids were collected, washed with advanced DMEM (Gibco) and embedded in Matrigel. The hIOs were cultured in advanced DMEM containing 1% of penicillin/streptomycin, 2 mM of L-glutamine (Gibco), 15 mM of HEPES (Gibco), 1 \times B27 (Gibco), 100 ng/mL of EGF (R&D Systems), 100 ng/mL of noggin (R&D Systems) and 500 ng/mL of R-spondin 1 (PeproTech). The cells were mechanically dissociated and re-embedded every 10-14 days. For in vitro maturation of hIOs, when the spheroids were embedded, 1 ng/mL of IL-2 (R&D systems) was added to the culture medium.²⁰ IL-2 was added freshly after at least two passages.

2.2 | Isolation and identification of bacteria

Lactobacillus strains were isolated from 1 g of fecal samples diluted in 10 mL of sterilized 0.85% of PBS (Corning) solution. Serially diluted fecal supernatants were spread on Man, Rogosa, and Sharpe (MRS, BD, Franklin Lakes, NJ, USA) agar plates and incubated in an anaerobic chamber containing 5% of H₂, 10% of CO₂, 85% of N₂ at 37°C. After 48 hours, a single ivory colony was picked and identified by 16S rRNA gene sequencing. Bacterial genomic DNA was extracted as described previously,²⁷ and the 16S rRNA gene sequence was amplified using universal primers. To ascertain the phylogenetic position of the isolates, the 16S rRNA gene sequences were compared with sequences obtained from the EzBioCloud (EzBioCloud (<https://www.ezbiocloud.net/>) and GenBank/EMBL/DDBJ (<http://www.ncbi.nlm.nih.gov/blast>) databases.

2.3 | Bacterial culture

Lactobacillus rhamnosus and *Lactobacillus reuteri* were cultivated in MRS agar at 37°C in anaerobic chamber. After 24 hours, a single colony was picked, seeded into MRS medium and incubated at 37°C in anaerobic chamber. mCherry-expressing *L. plantarum* (kindly donated by Won-Jae Lee at Seoul National University) were cultivated on MRS agar containing 5 ng/mL of erythromycin (Sigma-Aldrich, St. Louis, MO, USA).

2.4 | Microinjection of lactobacilli into hIOs and viable count assay

For microinjection, freshly cultured bacteria were collected, washed with PBS, and diluted to $1.5\text{--}2 \times 10^5$ cells/ μ L. After Matrigel dome disruption, the hIOs were washed and re-embedded into a new Matrigel dome in hIO culture medium without antibiotics. For stabilization, the organoids were incubated at 37°C for 24 hours. To prepare microcapillary tips for microinjection, 1-mm-diameter borosilicate glass with filament (Sutter Instrument, Novato, CA, USA) was pulled using a Micropipette Puller (Sutter Instrument) and cut to fine point. One microliter of bacterial suspension was loaded into the glass tip and injected into a hIO using Cell Tram 4r Oil (Eppendorf, Hamburg, Germany) controlled via a micro-manipulation system (Eppendorf). The injected hIOs were incubated at 37°C for 0, 6, or 12 hours in a CO₂ incubator or an anaerobic chamber. The hIOs were imaged using a Stereoscopic Zoom microscope (SMZ 745T; Nikon, Tokyo, Japan). To visualize microinjection, the bacteria were mixed with 0.4% (v/v) of trypan blue solution (Sigma-Aldrich). After incubation, microinjected hIOs were washed with PBS to remove residual bacteria and dissociated with trypsin-EDTA (Gibco). The cells were resuspended in 100 μ L of PBS and spread on an MRS agar plate. The bacteria were cultivated at 37°C in an anaerobic chamber for 24 hours and the CFU were counted. To evaluate bacterial growth in MRS and hIO media, 1×10^5 cells of *Lactobacillus* strains were cultivated in 10 mL of MRS and hIO media under anaerobic and aerobic conditions, and CFU were counted at 0, 6, 12, and 24 hours.

2.5 | Scanning electron microscopy

Attachment of lactobacilli to the luminal epithelia of the hIOs was detected by Scanning electron microscopy (SEM) as previously described,²⁸ and SEM images were acquired under a voltage of 15 Kv, using Inspect F50 equipment (FIE, Lausanne, Switzerland). Briefly, hIOs were washed with PBS, fixed in 2.5% of glutaraldehyde (Sigma-Aldrich) solution at 4°C for 2 hours, an post-fixed in 2% of osmium tetroxide (Sigma-Aldrich) at 4°C for 2 hours. The hIOs were dehydrated with 30%, 50%, 70%, 80%, 90%, and 100% of ethanol and washed twice with PBS. Then, they were immersed in hexamethylene diisocyanate (HMDS, Sigma-Aldrich) for 3 minutes and dried. Next, the hIOs were attached onto adhesive carbon tape and sputter-coated with gold at 10 mA for 60 seconds.

2.6 | Quantitative real-time PCR

Total RNA was extracted from organoids using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed

using a Superscript IV First-Strand Synthesis System kit (Thermo Fisher Scientific, Waltham, MA, USA) as described previously.²⁹ PCRs were run in a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Target gene expression was normalized to GAPDH expression. The primers used are listed in Table S1. Human small intestine total RNA was purchased from Clontech Laboratories, Inc (Mountain View, CA, USA) and used as a positive control.

2.7 | Immunofluorescence staining

hIOs microinjected with lactobacilli were fixed in 4% of paraformaldehyde (Sigma-Aldrich) and cryostored in 10%-30% of sucrose solution at 4°C overnight. The hIOs were embedded in optimal-cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and then cut into 10-μm sections. The sections were permeabilized with 0.1% of Triton X-100 in PBS for 20 minutes, blocked with 4% of bovine serum albumin solution at room temperature for 1 hour, and incubated with primary antibodies (Table S2) at 4°C overnight. After three washes with 0.05% of Tween-20 in PBS, fluorescence-labeled secondary antibodies (Table S2) were added and the sections were incubated at room temperature for 1 hour. The sections were washed thrice with 0.05% of Tween-20 in PBS, stained with 4',6-diamidino-2-phenylindole to visualize nuclei, and mounted with Fluorescence Mounting Medium (Dako, Kyoto, Japan). The sections were observed and imaged using an LSM800 confocal microscope (Carl Zeiss, Gottingen, Germany). To analyze the fluorescence intensity, real-time images were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8 | Alcian blue/periodic acid-Schiff staining

Cryosections of the OCT compound-embedded hIOs were used for the detection of the mucus layer. Alcian blue/periodic acid-Schiff staining was performed using alcian blue (Abcam, Cambridge, UK) and periodic acid-Schiff (Merck Millipore, Middlesex County, MA, USA) kits, following the manufacturers' recommended protocols and methods described previously.³⁰

2.9 | Microarray analysis

Microarray analysis was carried out using a Low RNA Input Linear Amplification Kit, a cRNA cleanup module, a one-color platform (Cy3), and a Whole Human Genome Microarray 4 × 44 K (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions and

as described previously.²⁰ Gene expression was evaluated using GeneSpring GX 7.3 (Agilent Technologies), and a cut-off of 2-fold change was used to determine differential expression. The scanned microarray images were normalized to the signals from undifferentiated hPSCs.

2.10 | Statistical analysis

All experiments were repeated at least three times. The actual replicate values are indicated in each figure legend. The data are represented by the mean ± standard deviation (SD). The colony-forming unit (CFU) assay was repeated more than three biological replicates, with technical triplicates. Thus, these data are represented by the mean ± standard error (SEM). Statistical analysis between groups or among multiple groups was determined by a two-tailed *t* test or one-way analysis of variance (ANOVA) followed by Tukey's post-test, respectively. Significance on each graph is represented as follows: ns; not significant, **P* < .05, ***P* < .01, ****P* < .001.

3 | RESULTS

3.1 | hPSC-derived hIOs support luminal growth of *Lactobacillus* spp

hIOs differentiated from hPSCs expressed various intestinal cell type-specific markers, including lysozyme (LYZ) for Paneth cells, villin 1 (VIL) for enterocytes, mucin 2 for goblet cells (MUC2), chromogranin A (CHGA) for enteroendocrine cells, LGR5 and SOX9 for intestinal stem cells, and KI67 as a proliferative marker. Further, hIOs had reduced expression of pluripotency markers such as *OCT4* and *NANOG* (Figure 1A,B). To assess whether the hIO lumen can serve as a mimic for apical enteric colonization of *Lactobacillus* spp., we microinjected hIOs with *L. rhamnosus* and *L. reuteri* cells. Microinjection of a hIOs with a mixture of a *Lactobacillus* suspension and trypan blue dye caused swelling, demonstrating luminal integrity (Figure 1C). *L. rhamnosus* and *L. reuteri* could be cultured under aerobic and anaerobic conditions, although anaerobic culture was more efficient (Figure 1D). However, hIO medium did not support the growth of these lactobacilli (Figure 1D). To evaluate epithelial colonization by *Lactobacillus* spp., we microinjected *L. rhamnosus* and *L. reuteri* grown in de MRS medium into the luminal cavities of hIOs. At 6 and 12 hours post-microinjection, we detected *L. rhamnosus* and *L. reuteri* cells in the hIO lumen, close to the microvilli, by scanning electron microscopy (SEM) (Figure 1E). There were more rod-shaped lactobacilli in the lumens of hIOs cultured in hIO medium under conditions that did not favor lactobacilli growth at 12 hours than at 6 hours

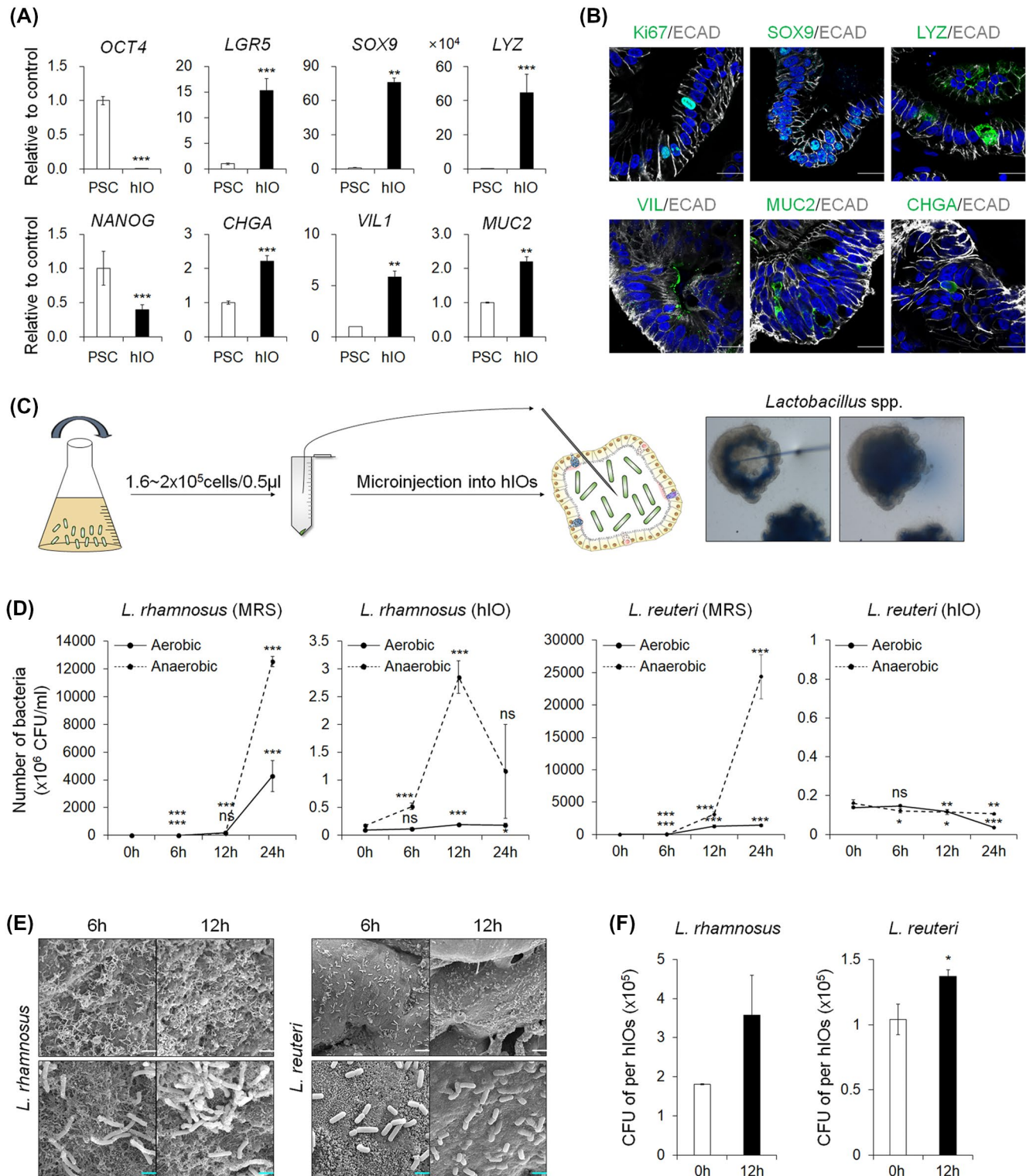
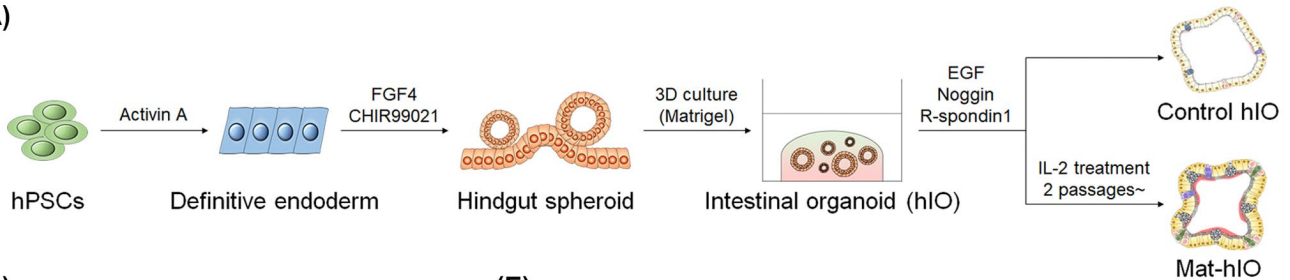


FIGURE 1 *Lactobacillus* spp. can grow inside hIOs derived from hPSCs. A, qPCR analysis of pluripotency markers (*OCT4*, *NANOG*) and intestinal cell type-specific markers (*LGR5*, *SOX9*, *VIL*, *LYZ*, *CHGA*, *MUC2*) in undifferentiated hPSCs and differentiated hIOs derived from hPSCs. Data are the mean \pm SD for $n = 3$ per group. *** $P < .001$, ** $P < .01$, * $P < .05$ according to t -test. B, Representative immunofluorescence staining of the Ki67 proliferation marker and intestinal cell type-specific markers. Scale bar, 20 μ m. C, Schematic diagram and bright-field images of microinjection of *Lactobacillus* spp. into hIOs. D, Aerobic and anaerobic growth of *L. reuteri* and *L. rhamnosus* in MRS and hIO media. Data are the mean \pm SD for $n = 3$ per group. *** $P < .001$, ** $P < .01$, * $P < .05$ according to t -test. E, Representative SEM images of luminal epithelia of hIOs at 6 and 12 hours after microinjection with *L. reuteri* and *L. rhamnosus*. Scale bar, white 10 μ m and blue 2 μ m. F, CFU recovered per hIO at 0 and 12 hours post microinjection ($n = 3$ per group). *** $P < .001$, ** $P < .01$, * $P < .05$ according to t -test.

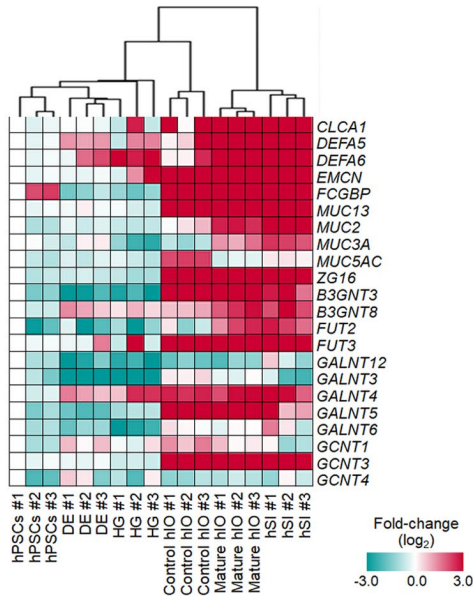
post-microinjection (Figure 1E). To quantitatively analyze microbial growth, luminal contents were cultured on MRS agar plates for colony formation assays. Immediately following microinjection, the mean colony-forming units per hIO

(CFU/hIO) for *L. rhamnosus* and *L. reuteri* were 1.8×10^5 and 1.0×10^5 , respectively (Figure 1F). We chose a time point of 12 hours after microinjection to amplify the difference in viability within hIOs. By 12 hours post microinjection,

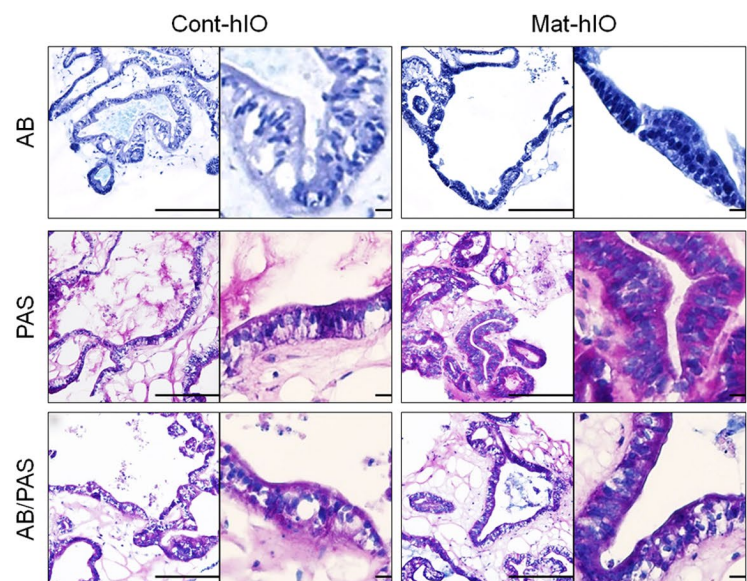
(A)



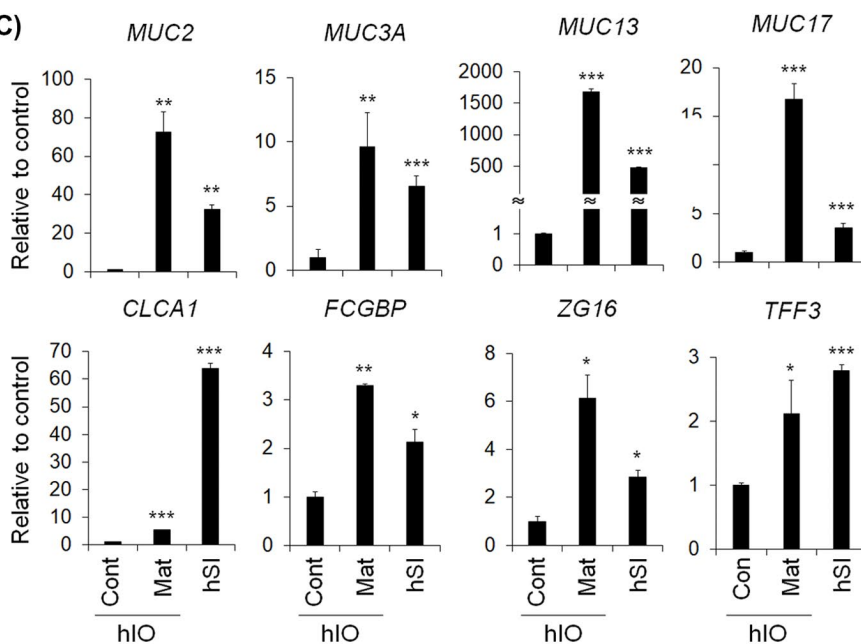
(B)



(E)



(C)



(D)

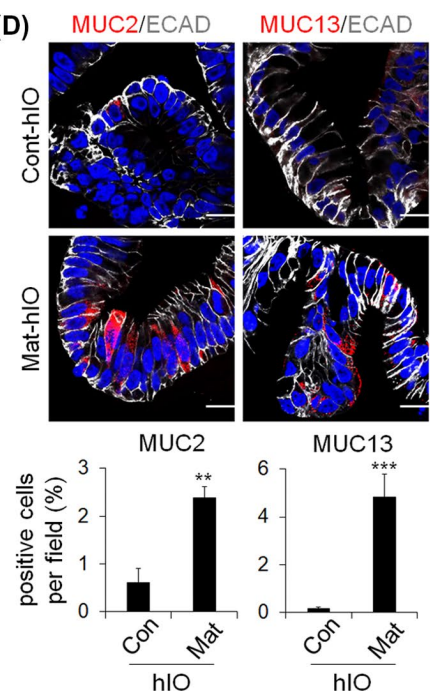


FIGURE 2 Mature hIOs have a thick and complex mucosal epithelium. A, Schematic representation of the in vitro maturation procedure for generating adult-like hIOs. B, Expression heatmap of genes encoding mucins and glycotransferases based on microarray data of undifferentiated hPSCs, definitive endoderm (DE), hindgut (HG), control and mature hIOs, and human small intestine (hSI) ($n = 3$ per group). Gene expression was normalized to undifferentiated hPSC #1 (shown in white). Magenta indicates upregulation, turquoise indicates downregulation. C, qPCR analysis of genes encoding mucins and glycotransferases in immature (control) and mature hIOs and hSI. Data are the mean \pm SD for $n = 3$ per group). *** $P < .001$, ** $P < .01$, * $P < .05$ according to one-way ANOVA. D, Immunofluorescence staining and quantification analysis of a secreted mucin marker (MUC2), a membrane-tethered mucin marker (MUC13), and an epithelial marker (ECAD) in immature and mature hIOs. Scale bar, 100 μm . Data are the mean \pm SD for $n = 3$ per group. *** $P < .001$, ** $P < .01$ according to t -test. E, Alcian blue (AB), periodic acid-Schiff (PAS), and combined (AB-PAS) staining for histological analysis. Scale bar, 50 μm

the mean CFU/hIO for *L. rhamnosus* and *L. reuteri* were 3.6×10^5 and 1.4×10^5 , respectively (Figure 1F), suggesting that *Lactobacillus* spp. can persist within the hIOs for at least 12 hours.

3.2 | The integrity of mucosal surfaces of mature hIOs is similar to that of their in vivo small intestinal counterpart

Our previous studies showed that early-passage hIOs derived from hPSCs, including hESCs and human-induced pluripotent SCs, can acquire a mature intestinal phenotype upon treatment with IL-2 for at least two passages (Figure 2A). Only after maturation in vitro did hIOs express the intestinal maturation marker genes *DEFA5*, *OLFM4*, *DPP4*, *LCT*, *KRT20*, *SLC5A1*, and *CREB3L3* (Figure S1). Various mucins are critical to intestinal mucosal integrity, which allows sustained colonization by intestinal microbiota.³¹ The mucins of the mucus layer are heavily glycosylated through various post-translational modifications mediated by glycotransferases.³² Therefore, we investigated the expression of genes encoding mucins and glycotransferases in immature (control) and mature hIOs. As differentiation proceeded, undifferentiated hPSCs gave rise to hIOs via differentiation into definitive endoderm (DE) and hindgut (HG), and the expression levels of these genes gradually increased (Figure 2B). The mucin and glycosyltransferase expression profile of mature hIOs was similar to that of adult human intestine (hSI) as indicated by microarray analysis (Figure 2B). This finding was supported by quantitative real-time PCR (qPCR) results for major secreted and membrane-bound GI mucins and mucin-related genes, including *MUC2*, *MUC3A*, *MUC13*, *MUC17*, *CLCA1*, *FCGBP*, *ZG16*, and *TFF3* (Figure 2C). We also examined the expression of MUC2, the major secreted gel-forming mucin forming a protective mucus barrier,³³ and MUC13, a cell- membrane-anchored mucin that protects the mature intestine against inflammation, by immunofluorescence staining (Figure 2D).³⁴ In mature hIOs, MUC2-positive cells were $2.38 \pm 0.22\%$ and MUC13-positive cells were $4.84 \pm 0.95\%$, whereas few cells were stained in immature hIOs ($0.61 \pm 0.29\%$ MUC2-positive cells, $0.17 \pm 0.06\%$ MUC13-positive cells) (Figure 2D). Alcian blue (AB) and periodic acid-Schiff (PAS) staining of sections from

immature and mature hIOs discriminated acid mucins (blue) within functional goblet cells from glycoproteins and neutral mucins (magenta) (Figure 2E). Importantly, the epithelia of mature hIOs produced a more robust and thicker mucus layer consisting of acidic and neutral glycoprotein components than did those of immature hIOs, suggesting that mature hIOs produce more mucins with modifications associated with microbial colonization than immature hIOs.³⁵⁻³⁷

3.3 | Mature hIOs have improved epithelial integrity and tight junctions

Next, we evaluated the luminal surfaces of hIOs by SEM. Mature hIOs displayed regular, densely packed apical microvilli, and had more villi per unit area than immature hIOs (Figure 3A). Tight junctions contribute to intestinal epithelial integrity and cell polarity.³⁸ Therefore, we examined the expression of tight junction molecules in immature and mature hIOs by qPCR and immunofluorescence analyses. In mature hIOs, *zonula occludens-1* (*ZO-1*), *occludin*, *claudin 1*, *claudin 3*, and *claudin 5* mRNA levels were higher than in control hIOs, indicating that mature hIOs are more similar to adult hSI (Figure 3B). Mature hIOs showed markedly higher expression of ZO-1 and claudin1 in the epithelial layer than control hIOs (Figure 3C). These results suggested that mature hIOs show greater similarity to adult hSI than to fetal and neonatal intestine in terms of mucus composition and epithelial structure.

3.4 | Mature hIOs allow better growth and colonization of lactobacilli than do immature hIOs

Whether hIO maturation affects lactobacilli growth and colonization was evaluated using high-resolution SEM images. Rod-shaped, 4- μm -long *L. reuteri* cells were observed on the luminal epithelial surfaces of immature and mature hIOs at 6 and 12 hours post-microinjection. However, *L. reuteri* cells were more numerous in mature than in immature hIOs (Figure 4A). When control and mature hIOs injected with *L. reuteri* cultured in hIO medium were disrupted and plated on MRS agar medium, mature hIOs produced significantly

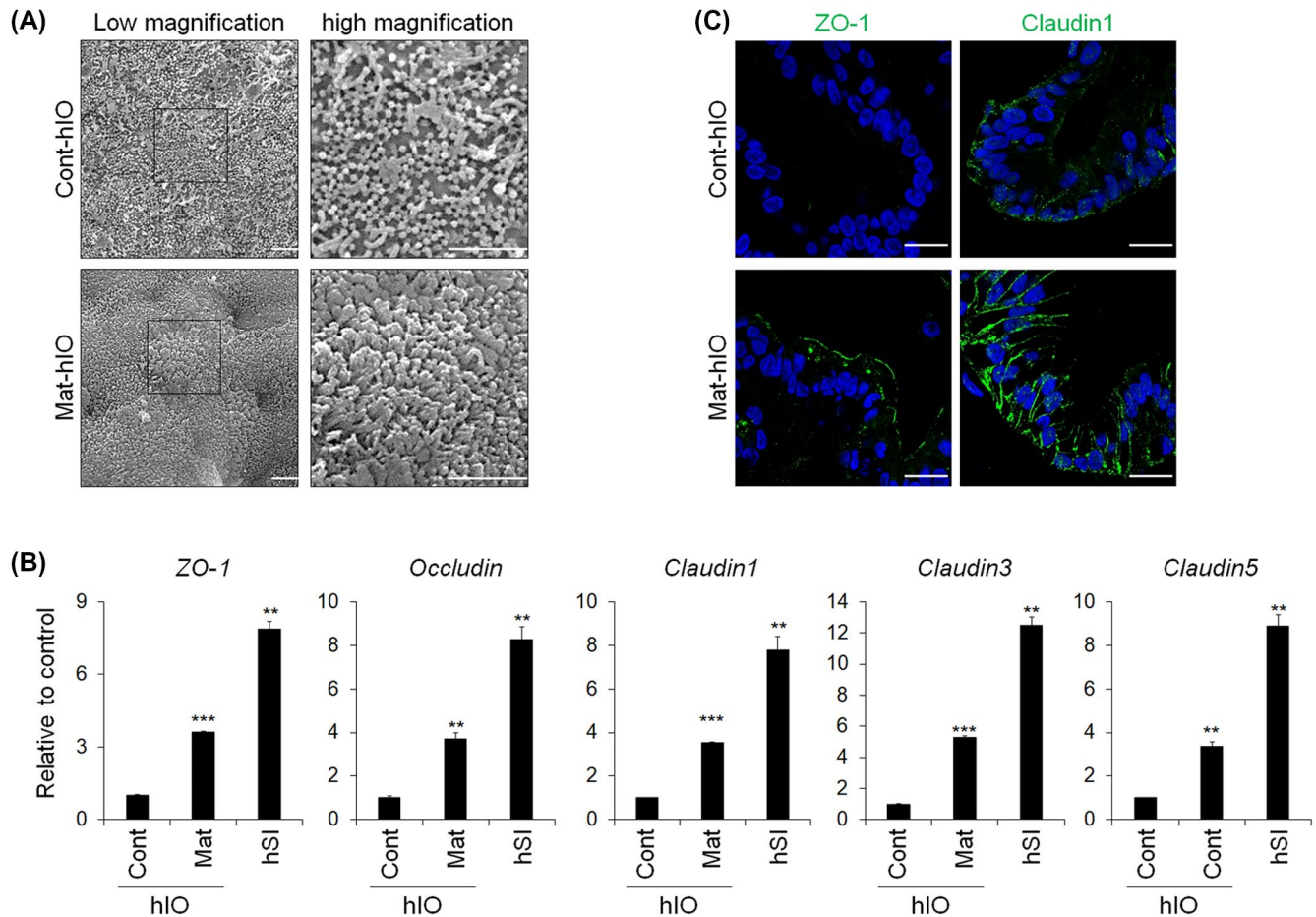


FIGURE 3 Mature hIOs exhibit improved villus morphology and tight junction integrity on the luminal surface. A, Representative SEM images showing villi- and microvilli-like structures on the luminal surfaces of intestinal epithelial cells inside hIOs. Scale bar, 2 μ m. B, qPCR of tight junction markers (ZO-1, occludin, claudins 1, 3, and 5) in immature and mature hIOs and hSI. Data are the mean \pm SD for $n = 3$ per group. *** $P < .001$, ** $P < .01$ according to one-way ANOVA. C, Immunofluorescence staining of tight junction markers (ZO-1 and claudin 1) in immature and mature hIOs. Scale bar, 20 μ m. *** $P < .001$, ** $P < .01$, * $P < .05$ according to t -test

more colonies (Figure 4B,C), which indicates that mature hIOs had more mucous component, which might serve as nutrients for the lactobacilli. To evaluate the significance of hIO maturation quantitatively, control and mature hIOs were injected with *L. plantarum* constitutively expressing red fluorescent protein (RFP; mCherry) and imaged using a real-time microscope. Mature hIOs microinjected with 2.7×10^6 live *L. plantarum* cells expressing RFP exhibited significantly more intense red fluorescence than did control hIOs within 12 hours post-microinjection (Figure 5A,B, Movies S1 and S2). Numerous *L. plantarum* localized to the luminal spaces of mature hIOs at 12 hours post-microinjection and were observed at the apical epithelial surfaces of hIOs (Figure 5C). When intraluminal *L. plantarum* were collected from control and mature hIOs 12 hours post-microinjection, disrupted and plated on MRS agar medium, the number of viable bacteria in mature hIOs was increased compared to control hIOs (Figure 5D,E), suggesting that the intensity of fluorescence signal from RFP-expressing *L. plantarum* cells was consistent with viable cell count analysis.

4 | DISCUSSION

Lactobacillus spp., which mainly colonize the vaginal tract and the small intestine, have health-promoting effects.³⁹⁻⁴¹ Even though *Lactobacillus* spp. are not dominant species in the adult GI tract, these species form the core intestinal microbiota in the infant intestine due to the large amount of *Lactobacillus* derived from the maternal vaginal tract^{42,43} or breast milk^{44,45} during infancy. Various factors derived from *Lactobacillus* spp. are very important to early development, maturation, intestinal maintenance and regeneration, immune system activation, and prevention of pathogenic colonization.⁴⁶⁻⁴⁸ Additionally, *Lactobacillus*-derived metabolites such as short chain fatty acids regulate epithelial barrier and tight junction integrity, enhance mucus release, and promote differentiation in intestinal epithelial cells.^{49,50} Efficient and proper adherence of lactobacilli to the intestinal epithelium is important for persistent colonization, stimulation of microbe-host epithelial interactions *via* immunomodulation, and epithelial barrier integrity for protection against pathogens.⁵¹

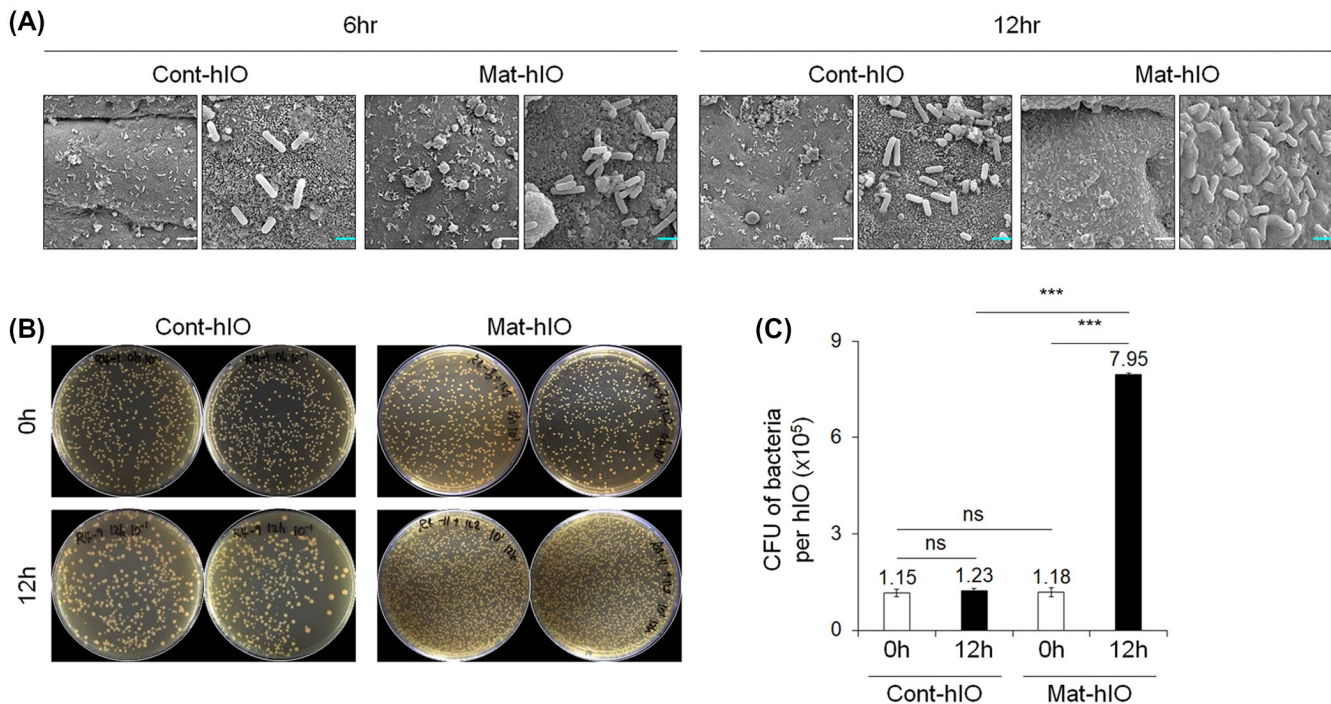


FIGURE 4 In vitro maturation of hIOs promotes the growth of and colonization by *L. reuteri*. A, Representative SEM images of immature and mature hIOs at 6 and 12 hours after microinjection with *L. reuteri*. Scale bar, white 10 μ m and blue 2 μ m. B, Colonies of *L. reuteri* on de Man, Rogosa, and Sharpe agar plates. C, luminal CFU recovered from hIOs after manually cutting the hIOs at 0 and 12 hours post microinjection in immature and mature hIOs, respectively. Data are the mean \pm SEM for n = 5 per group. ****P* < .001, according to one-way ANOVA

However, appropriate small intestinal models to study commensal microbial colonization in humans have not been developed. Though high-throughput nucleotide sequencing has revealed the identity and abundance of intestinal microbial communities, the molecular roles and mechanisms of action of specific organisms present on the human intestinal epithelium remain unclear.⁵² In this study, we established a model to mimic commensal bacterial colonization of the apical surface of the intestinal epithelium, using microinjection of small intestinal organoids. These hPSC-derived hIOs recapitulate the cellular composition and functions of the small intestinal epithelium. Microinjection of lactobacilli into the hIO lumen resulted in stable growth on and colonization of the luminal surface of the hIO (Figure 1). Furthermore, the in vitro-matured hIOs more efficiently sustained the lactobacilli than did immature hIOs (Figures 4 and 5), which was attributed to a more developed mucus layer (Figure 2).

The development of a healthy mucosa, particularly the host-derived mucus layer, is important for persistent small intestinal colonization by commensal microbiota as it provides attachment sites and serves as a substrate for the growth of beneficial microbes.^{41,53} While dietary carbohydrates represent a main energy source for the intestinal microbiota, host-derived glycans, such as intestinal mucins, which are highly glycosylated O-linked glycoproteins, constitute a major carbon and energy source for intestinal microorganisms and contribute to commensal bacterial colonization, survival,

and persistence in the human intestine.⁵⁴ Mature hIOs produced a robust and dense mucus layer around the villus tips in the luminal surface that contained neutral and acidic mucins (Figure 2). Tight junctions, particularly tight junction-related proteins in the intestinal epithelial layer, also contribute to mucosal barrier integrity.⁵⁵ Tight junction disruption may be responsible for microbe-associated diarrhea.³⁸ Mature hIOs exhibited more complex and complete tight junctions than did immature hIOs based on tight junction-related gene and protein expression (Figure 3). To more closely investigate epithelial transcriptomic changes, the expression of genes encoding secreted and membrane-associated mucins and mucin post-translational modification-related glycotransferases was analyzed using microarray data. Mature hIOs exhibited transcriptional activation of various mucins and glycotransferases, suggesting that in vitro maturation broadly affects the molecular and cellular characteristics of the intestinal epithelium in hIOs (Figure 2B). Therefore, mature hIOs, having improved intestinal villi integrity and tight junction structure, provide an effective system for studying host-microbe interactions at the intestinal interface.

One of the practical barriers to studying the microbiota using organoids is that most microbiota are anaerobic, whereas adult tissue-derived epithelial organoids and hPSC-derived hIOs are cultured under normal oxygen conditions in vitro.¹² However, *Lactobacillus* spp., traditionally classified as oxygen-tolerant bacteria, can be cultivated under

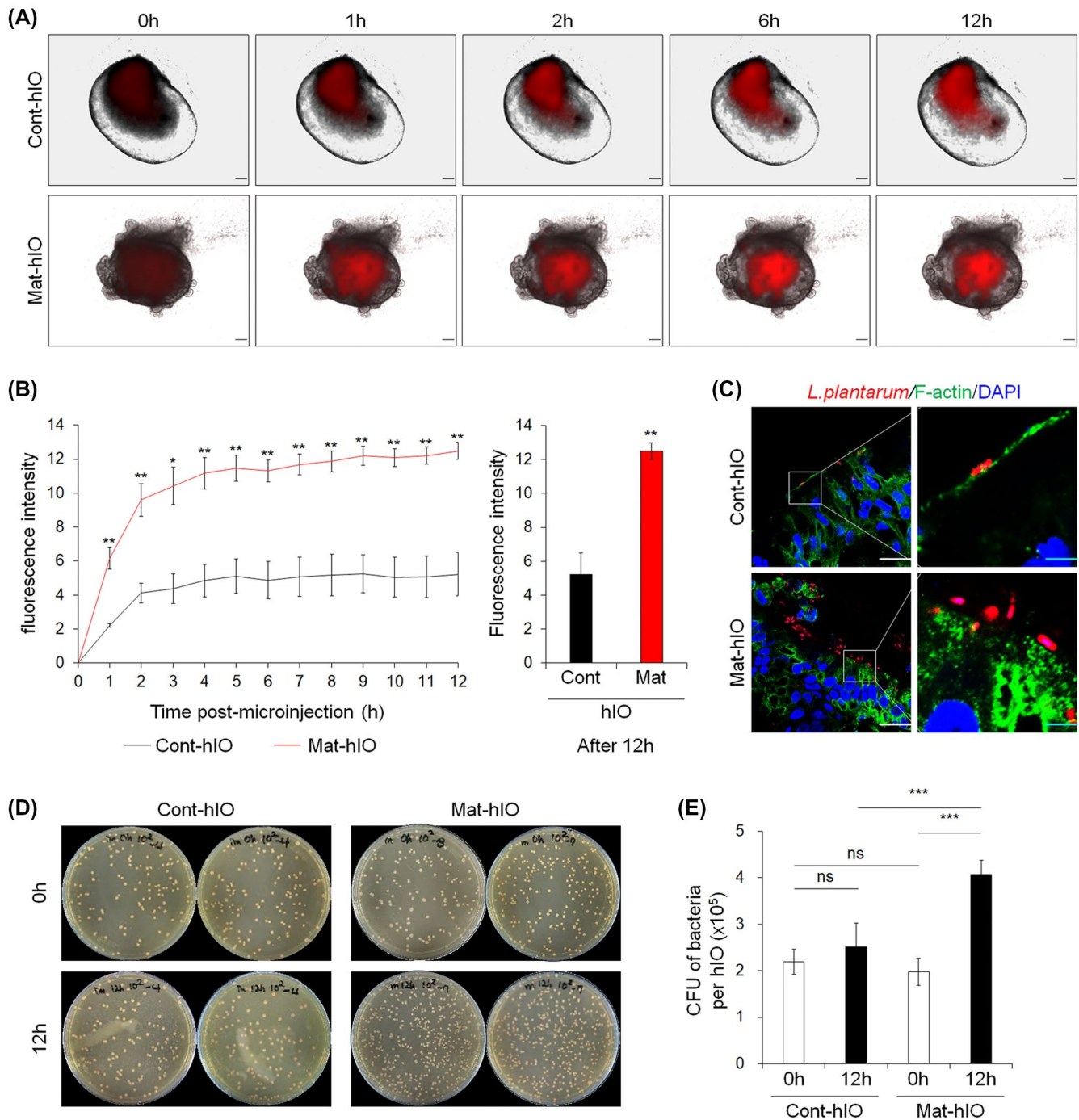


FIGURE 5 Mature hIOs more efficiently support luminal growth of *L. plantarum*. A, Representative time-lapse images of immature and mature hIOs after microinjection with RFP⁺ *L. plantarum*, taken at the indicated time points from videos (Movies S1 and S2). Scale bar, 200 μ m. B, Mean fluorescence intensity of immature and mature hIOs containing live RFP⁺ *L. plantarum* cells (F₁-F₀). C, Confocal micrograph of the epithelium (F-actin, green; nuclei, blue) of immature and mature hIOs in direct association with RFP⁺ *L. plantarum* at 12 hours post microinjection with 2.7×10^6 live *L. plantarum* cells. Scale bar, white 20 μ m and blue 4 μ m. Data are the mean \pm SE. ** $P < .01$, * $P < .05$ according to t-test. D, Colonies of *L. plantarum* on de Man, Rogosa, and Sharpe agar plates. E, luminal CFU recovered from hIOs after manually cutting the hIOs at 0 and 12 hours post microinjection in immature and mature hIOs, respectively. Data are the mean \pm SEM for n = 5 per group. *** $P < .001$, according to one-way ANOVA

the aerobic culture conditions⁵⁶ used for culturing hPSC-derived hIOs. Therefore, mature hIOs mimicking the human adult small intestine can be optimized for the study of commensal lactobacilli, which mainly inhabit the small intestine. In addition, our previous study showed that intestinal

Paneth cells in mature hIOs are able to secrete the microbial α -defensins HD-5 and HD-6.^{20,57} Considering that α -defensins secreted from mouse small intestinal organoids can kill bacteria,⁵⁸ it can be assumed that in vitro-matured hIOs can be used to observe interactions between the small intestinal

epithelium and various enteric pathogenic bacteria. However, to study host immune response-microbe interactions, mature hIOs need to be further developed as they lack components of the human immune system, which comprises an essential part of the host response to commensal microbiota and infection.⁵⁹ The complex and dynamic interactions among the intestinal epithelium, the immune system, and microbes is essential to understand the physiology of the small intestine.⁵²

The lack of appropriate experimental models has hampered research on human commensal microbiota, such as *Lactobacillus* spp. Recently, human enteroids and hPSC-derived immature hIOs have been mainly utilized to study host-pathogenic microbe interactions.¹³ However, the models used in previous studies resemble the fetal, immature intestine, rather than the mature intestine. This study revealed that mature hIOs better mimic the adult intestine. In addition, to the best of our knowledge, this is the first study to demonstrate that hIOs can be stably colonized with human commensal lactobacilli.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

Y.S. Son performed the molecular and differentiation experiments for the hIOs; S.J. Ki and C.-M. Ryu performed the microbial experiments; R. Thanavel performed and analyzed the scanning electron microscopy experiments; J.-J. Kim performed the molecular experiment; M.-O. Lee, J. Kim, C.-R. Jung, T.-S. Han, H.-S. Cho, and C.-M. Ryu analyzed the data and critically revised the manuscript; M.-Y. Son, D.-S. Park, and S.-H. Kim planned the project, designed the studies, analyzed the data and wrote the manuscript. All authors helped to write and approved the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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