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IN VITRO MODEL SYSTEMS FOR HOST-MICROBIOTA INTERACTION STUDIES

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Abstract – Despite the efforts to explore the communication between the human gut and intestinal microbiota in recent years, the complexity of this interaction remains not fully understood. This is mostly due to a lack of mechanistic insights into how this interspecies relationship works. However, ongoing work in the employment of various in vitro systems provides an excellent opportunity to understand the role of different components in host-microbiota signaling. This work provides a structured review of the most used in vitro model systems for the exploration of host-microbiota interactions in health and disease published in the last year. The data presented uncovers the variety of available models, such as immortalized cell monocultures, 3D epithelial organoids and their monolayers, co-cultures, gut-on-a-chip platforms, scaffolds, etc., and their applications in different scientific contexts. Future studies are needed to deepen the understanding of the interplay between gut mucosa and microbial communities, improve the existing in vitro systems, and develop new experimental platforms.

Keywords: Model system, Host-microbiota interaction, Microbiota, Organoids, Cell cultures.

INTRODUCTION

Extensive research has shown that commensal microbes and their interaction with the host are associated with immune system development[1,2], aging[3], health and disease[4,5], and the effectiveness of therapeutic interventions[6,7]. The key components of this complex interspecies relationship are microbial communities, intestinal epithelium, and immune system. Understanding the complex dynamics between host and associated microbiota has become a critical area of research. *In vitro* systems provide a controlled environment to study microbial communities and their interactions with host tissues, offering insights that are often out of reach *in vivo* due to animal and human studies' complexity and ethical concerns. These models range from simple monocultures to sophisticated microfluidic devices that mimic the three-dimensional architecture and microenvironment of host tissues[8,9]. They provide valuable insights into microbial colonization, immune responses, and metabolic interactions. Recent methodological advances have enhanced the physiological relevance of these models, bridging the gap between *in vitro* findings and biological processes.

METHODS

In this review, we provide an overview of the most recent work published during the past year (from March 2023 to March 2024) on *in vitro* model systems for host-microbiota interaction studies. Based on the model system used in the reviewed literature we distinguished

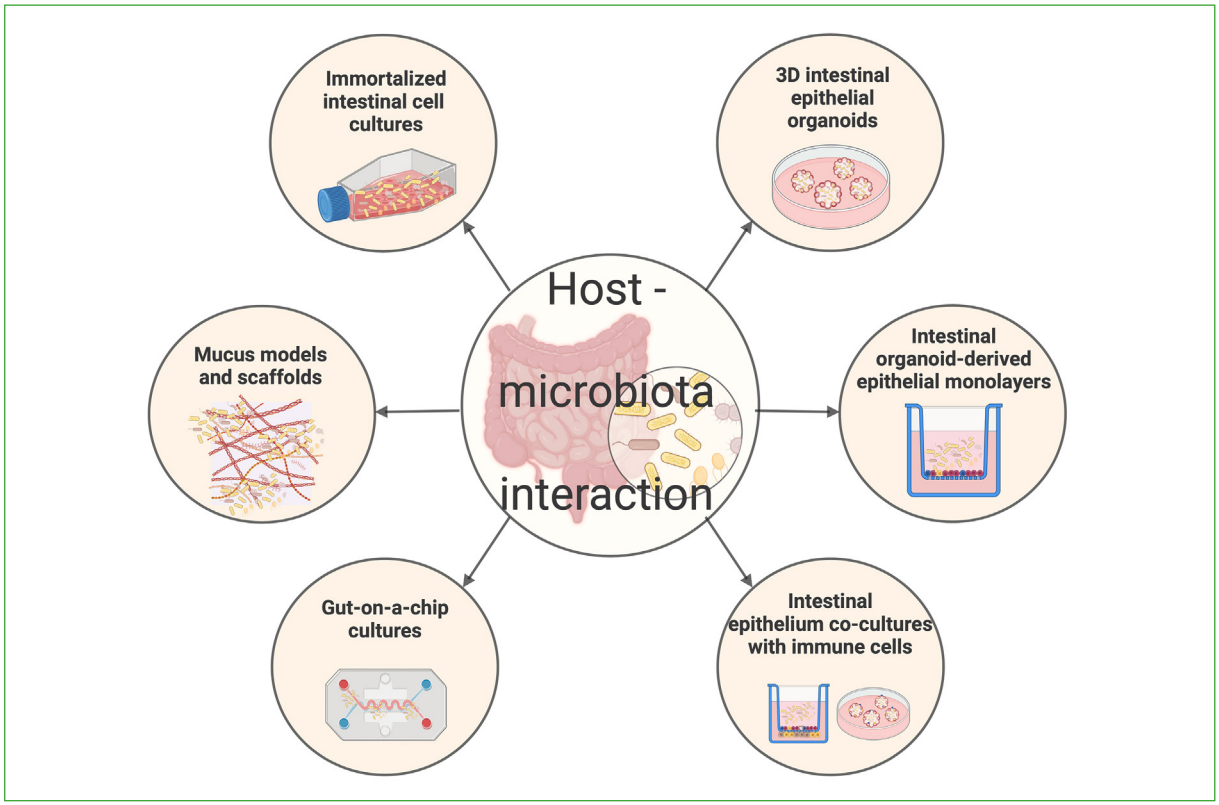

Figure 1. The diversity of *in vitro* model systems for host-microbiota interaction studies. Created with BioRender.com.

six methodological groups (Figure 1) and described the key findings as well as highlighted emerging novel model systems and variations for the investigation of host-microbiota interactions.

IMMORTALIZED CELL CULTURE MODEL

Immortalized cell lines remain the cornerstone of *in vitro* studies. They are easy to culture, cost-effective, and scalable. Furthermore, very recent studies provide important insights into the research of novel probiotics or microbiota-derived molecules.

Recent study by Torkamaneh et al[10] presented results of the *in vitro* assay that aimed to assess the effects of isolated probiotics on autophagy signaling pathways in the HT-29 cell line. *Bifidobacterium* spp. probiotics were added to cell culture before, after, and simultaneously with inflammatory compounds, and the expression of eight genes involved in the signaling pathway of autophagy was evaluated. Authors demonstrated altered expression of *PIK3C3, BECLIN, ATG7, ATG5*, and *ATG16* genes in the pre- and simultaneous treatment group. Meanwhile, the expression of *ATG14* and *ATG3* genes was increased in the post-treatment group[10]. These findings indicate that *Bifidobacterium* spp. can prevent and reduce inflammation in the early stages of inflammation-related disorders. Microbiota-derived molecules show great potential as a source of pharmacological agents, and recent studies underscore the significance of bacteria-derived short-chain fatty acids for maintaining health[11,12]. Hamed et al[13] used a human colonic T84 epithelial cell line and showed that butyrate significantly reduced mitochondrial fragmentation and activated mitochondrial biogenesis after treatment with *E. coli*-LF8. A deeper investigation revealed that butyrate had no bacteriostatic or bactericidal effects, did not reduce bacterial invasion, and mitochondrial fragmentation was reduced *via* FFAR-3 receptor. In addition, butyrate affected the abundance of microbe-derived metabolites in *E. coli*, suggesting a potential butyrate role not only in epithelial cells but also in *E. coli*[13]. The summary of the reviewed results is shown in Table 1.

TABLE 1. SUMMARY OF *IN VITRO* MODEL SYSTEMS USED FOR HOST-MICROBIOTA INTERACTION STUDIES.

Culture system	Host cells	Bacteria/bacterial products	Key findings	Reference
Immortalized cell culture	Human colorectal adenocarcinoma cell line (HT-29)	*Bifidobacterium* spp. (*B. longum*, *B. infantis*, and *B. bifidum*) probiotics	Prevention and reduction of inflammation by *Bifidobacterium* spp.	[10]
	Human colonic epithelial cell lines (T84, Caco-2)	*E. coli*-LF82 pathobiont	Protective role of bacterial product butyrate against epithelial mitochondrial disruption caused by *E. coli*-LF82 *via* FFAR3.	[13]
	Mouse colon cancer cell lines (CT26, MC38)	*Coriobacteriaceae* species strain Cori.ST1911, *Lactobacillus* strain *La.mu*730	Promotion of colorectal tumorigenesis by *Cori*.ST1911 via the CPT1A-ERK axis; Inhibition of *Coriobacteriaceae* colonisation and reversion of its carcinogenic effect by *La.mu*730 strain.	[15]
	Human colorectal adenocarcinoma cell line (Caco-2), cervical carcinoma cell line (HeLa)	TcdA and TcdB toxins from *C. difficile* NAP1/027 strain	*Delayed C.*difficile intoxication and preservation of epithelial integrity by drug amiodarone.	[19]
3D intestinal organoids	Mouse intestinal organoids	*Coriobacteriaceae* species strain *Cori*.ST1911, *Lactobacillus* strain *La.mu*730	Promotion of colorectal tumorigenesis by *Cori*.ST1911 *via* the CPT1A-ERK axis; Inhibition of *Coriobacteriaceae* colonisation and reversion of its carcinogenic effect by *La.mu*730 strain.	[15]
	Piglet jejunum organoids	*B. amyloliquefaciens* SC06	Decrease in inflammatory response and promotion of epithelial barrier healing by *B. amyloliquefaciens* SC06.	[16]
	Mouse intestinal organoids (wild-type and propionate receptor-deficient)	Propionate generated by *B. thetaiotaomicron*	Promotion of goblet cell differentiation by *B. thetaiotaomicron* produce propionate via propionate receptor GPR41	[17]
	Mouse intestinal organoids	Tryptophan degradation product indole-3-carboxaldehyde (I3A)	Radioprotective effect of I3A by promotion of ISCs survival and intestinal epithelial cell proliferation through the AhR/IL-10/Wnt signaling pathway	[20]
	Human intestinal organoids	TcdA and TcdB toxins from *C. difficile* NAP1/027 strain	Protective effect of drug amiodarone against *C. difficile* toxins	[19]
	Human intestinal (duodenum, jejunum, ileum, and ascending colon) organoids	*L. reuteri* cell-free conditioned medium	Induction of oxytocin release by *L. reuteri via* secretin	[21]
	Mouse intestinal organoids	*D. vulgaris* flagellin	Promotion of colitis by *D. vulgaris via D. vulgaris* flagellin-LRRC19 interaction.	[20]

CONTINUED

TABLE 1 *(CONTINUED)*. SUMMARY OF *IN VITRO* MODEL SYSTEMS USED FOR HOST-MICROBIOTA INTERACTION STUDIES

Culture system	Host cells	Bacteria/bacterial products	Key findings	Reference
2D intestinal organoid monolayers	Human intestinal (duodenum and jejunum) organoid-derived epithelial cell monolayer	Probiotic bacterial strains LGG® , DSM33361, BB-12®, Bif195, and *Salmonella*	Development of an *in vitro* cell culture platform for epithelial cell-microbe interaction studies.	23
	Human intestinal (duodenum) organoid-derived epithelial cell monolayer	Bacterial pathogen *S. flexneri*	Development of human malnourished organoid model to study effects of nutrient deprivation on gastrointestinal epithelium (e.g., susceptibility toinfection).	25
	Monolayers of human colonic organoids	*E. coli*-LF82 pathobiont	Protective role of bacterial product butyrate against epithelial mitochondrial disruption caused by *E. coli*-LF82 *via* FFAR3.	13
	Monolayers of human colonic organoids	*E. coli*, *P. vulgatus*	Potential impairment of tight junction protein coding gene *ZO-1* in epithelial cells of patients with ulcerative colitis.	24
Co-cultures with immune cells	Mouse intestinal (jejunum) organoids	–	Development of an *in vitro* co-culture model of dendritic cells with small intestinal organoids.	26
	Monolayer comprising of Caco-2, HT29-MTX, T cells, B cell-derived factors	–	Development of a complex small intestine *in vitro* model. Suppression of macrophage/dendritic cells differentiation by small intestine epithelial cells.	27
Gut-on-a-chip and microfluidics	Caco-2 cell line, induced pluripotent stem cell-derived enteric neurons	*L. reuteri* F275	Establishment of NeuroHuMix system – a gut-on-a-chip model including enteric neuronal cells in co-culture with gut epithelial cells and bacteria, to study gut microbiome-nervous system axis.	28
	Epithelial cell monolayer generated from neonatal small intestine organoids and human intestinal microvascular endothelial cells (HIMECs)	Polymicrobial enteric bacteria culture from patient with severe necrotizing enterocolitis (NEC)	Development of NEC-on-a-chip model consisting of a microfluidic device seeded with intestinal enteroids in co-culture with human endothelial cells and microbiome from an infant with severe NEC.	29
	3D stratified gut epithelium derived from Caco-2 cells	*B. fragilis* (ETBF), *Lactobacillus* spp.	Establishment of a scalable Gut Microbiome-on-a-Chip (GMoC) with great imaging capability and scalability for the investigation of gut-microbiome interfaces.	30
	Human colorectal adenocarcinoma cell line (Caco-2)	*E. coli* K-12 MG1655-derived genetically modified strains (*E. coli* K-12 MG1655-ΔdecR, *E. coli* K-12 MG1655-ΔmalY, and *E. coli* K-12 MG1655-ΔsseA)	Development of a gut-on-a-chip model maintaining hydrogen sulfide gas tension and enabling real-time visualization to investigate the causal role of gaseous microbial metabolites.	31

CONTINUED

TABLE 1 *(CONTINUED)*. SUMMARY OF *IN VITRO* MODEL SYSTEMS USED FOR HOST-MICROBIOTA INTERACTION STUDIES.

Culture system	Host cells	Bacteria/bacterial products	Key findings	Reference
Mucus models	The fluorine-assisted mucus surrogate (FAMS), human colorectal adenocarcinoma cell line (Caco-2)	*E. coli*, *L. rhamnosus* and *L. acidophilus*	Fabrication of a self-assembling gel replicating the diverse mechanical, structural, and biochemical profiles of colonic mucus with the possibility to integrate with colorectal epithelial cells.	33
	Type II mucins from porcine stomach, containing MUC2	Fecal microbiota	Generation of a 3D mucin coated electrospun gelatin scaffold-based model with increased spatial complexity and better reproduction of mucosal environment.	35

PRIMARY CELLS AND ORGANOIDS

3D organoids

Intestinal organoids, derived from induced pluripotent stem cells or tissue-resident intestinal stem cells (ISCs), are powerful *in vitro* models mimicking the gut. The latter polarized 3D systems established from intestinal crypts can self-renew, and differentiate to intestinal epithelial cells, such as enterocytes, enteroendocrine cells, Paneth cells, and goblet cells, therefore retaining certain physiological functionality[14]. All these features make ISC organoids an attractive model for host-oriented research and precision medicine, superior to previously widely used monocultures, enabling the study of host-microbiome interactions with great experimental control.

In recent years, organoids have been widely employed in host-pathogen interaction studies, including direct co-culturing with specific bacterial strains. Tang et al[15] used mouse intestinal organoids to demonstrate that the endogenous *Lactobacillus* strain can interfere with *Coriobacteriaceae* colonization. *Lactobacillus* restored gut barrier function by reversing *Coriobacteriaceae*-induced activation of the CPT1A-ERK axis and destruction of mucin-related proteins, thus supplementing their investigation on new gut microbiota intervention strategies for treatment and prevention of colorectal cancer[15]. In the context of gut inflammation, interesting findings on the enhancement of ISCs proliferation and differentiation induction, as well as the maintenance of barrier integrity by *Bacillus amyloliquefaciens* SC06, were published by Wang et al[16] Co-culturing with *B. amyloliquefaciens* increased the size of organoids, the number of Lgr5+ stem cells and the expression of intestinal stem cells, goblet cells marker genes together with the expression of Wnt/β-catenin pathway components, supporting the *in vivo* findings on therapeutic regenerating functions of the investigated bacterial strain[16].

Several studies investigating the effects of bacterial products and their implications on gut epithelium and host response emerged recently. Wang et al[17] published a study investigating the effects of bacterial metabolite propionate on epithelial inflammation. Authors used organoids generated from wild-type and propionate receptor GPR41-deficient mice to confirm that *Bacteroides thetaiomicron* methylmalonyl-CoA mutase-mediated propionate biosynthesis promotes goblet cell development via GPR41 signaling, therefore, suggesting potential pathways to mitigate colitis[17]. Another study by Xie et al[18] demonstrated radioprotective properties of microbiota-dependent tryptophan degradation product indole-3-carboxaldehyde (I3A). I3A improved the rate of budding organoids and surface area of organoids, reduced the number of damaged organoids after ionizing radiation exposure, and promoted the damage repair of intestinal epithelial cells in an AhR/IL-10/wnt3/β-catenin pathway-dependent manner[18]. Application of other bacterial products, such as toxins of *Clostridioides difficile*, flagellin of

Desulfovibrio vulgaris or *Limosilactobacillus reuteri* conditioned media on 3D organoids have also been used to gain valuable insights into mechanisms behind protective effects of drugs, inflammation in colitis or modulation of hormone secretion in intestinal epithelial cells[19-21] (Table 1).

2D Intestinal Organoid Monolayers

3D intestinal organoids retain polarized epithelium resulting in a cystic shape with the inside corresponding to the gut lumen. To accurately represent microbiome-epithelium orientation for the investigation of host-microbiome interactions, microinjection of microbiota into the lumen of organoids is required. Although protocols for the inversion of organoid polarity exist[22], both generation of apical-out 3D organoids and microinjections of microbiota require sophisticated manipulations. 3D intestinal organoid-derived 2D epithelial monolayers cultivated in cell culture plates or Transwells can be used for host-microbiome interaction studies without the need for microinjections.

During the review period, several studies applied 2D organoid-derived primary epithelial cell models to investigate bacteria-epithelium interactions. Bornholdt et al[23] aimed to create a reproducible 2D *in vitro* cell culture platform mimicking the differentiated intestinal epithelium exposed to microbes *in vivo.* The authors optimized cell culturing methods (cell culture medium, extracellular matrix) and thoroughly described the epithelium transcriptional profile based on the interaction with probiotic and pathogenic bacterial strains. Interestingly, the results showed that while the developed primary epithelial cell system generated a robust response to microbes, Caco-2 cells showed little or no response to the same bacterial strains, thus showing that organoid-derived monolayer is a more sensitive platform for epithelium-microbe interaction studies compared to conventional models of immortalized cell lines[23]. A study by Hamed et al[13] (previously described in this review), used organoid-derived monolayers to confirm the protective role of butyrate in the restoration of mitochondrial damage caused by IBD-related pathobiont *E. coli*-LF82 and thereby provided valuable results on how the loss of butyrate-producing bacteria in IBD could contribute to the host susceptibility to pathobionts. In another IBD study, Inciuraite et al[24] used colonic epithelial cell and commensal *E. coli* or *Phocaeicola vulgatus* co-culture systems. By employing organoid-derived monolayers generated from 17 individuals, authors revealed a potential differential response in colonic epithelial tight junction formation caused by commensal microbiota members when comparing UC patients and non-IBD controls. Furthermore, by assessing patient-to-patient responses to both bacteria species, the authors uncovered a high inter-individual variation, reflecting UC patient-specific epithelial cell reactivity to normal gut microflora[24]. This latter observation emphasizes the need for more comprehensive research in the field. A foundational approach to organoid culturing was proposed by Perlman et al[25], as the group attempted to develop a 2D model of malnourished duodenal organoids by selectively limiting distinct macronutrients in organoid media. They further validated the system by confirming the expression of malnutrition biomarkers and reduced barrier integrity, which is critical to protecting the gastrointestinal tract from infection. To test the model, authors co-cultured malnourished organoids with the bacterial pathogen *Shigella flexneri* and found that malnourishment increased the ability of *S. flexneri* to invade the intestinal epithelium. This work developed a system with an altered nutritional state of organoids that could be an excellent platform for the investigation of the interaction between malnourishment and the host response to enteric pathogens[25] (Table 1).

Epithelial Co-Cultures With Immune Cells

Microbiota-host interaction mechanisms are inseparable from immune cell signaling *in vivo*. Therefore, intestinal epithelium-immune cell-bacteria co-culture models are being developed. Particular attention has been drawn to dendritic cells (DCs), which are specialized sentinel cells, participating in recognition and responses to gut microbiota. By using a novel 3D co-culture model of *in vitro* differentiated 'gut-like' DCs with small intestinal organoids, John-

ston et al[26] showed that intestinal epithelium regulates both retinaldehyde dehydrogenase activity in DCs (crucial to the regulation of adaptive immunity) and the extension of trans-epithelial dendrites into the luminal space (important for antigen transfer). Another study by Schimpel et al[27] reported a 2D model composed of three intestinal epithelial cell types (absorptive enterocytes, antigen-delivering microfold cells, and mucus-producing goblet cells) together with T cells and B cell-derived factors. Studies revealed that intestinal epithelial cells suppress monocyte/macrophage differentiation, while enterocytes and goblet cells may contribute to the promotion of intestinal DC and macrophage differentiation[27]. Such complex co-culture models could be utilized not only to grow an understanding of how epithelial and immune cells affect DCs and vice versa, but also to define communication between these components under a variety of physiological and pathological conditions, including exposure to bacteria (Table 1).

GUT-ON-A-CHIP AND MICROFLUIDICS

Efforts are ongoing to optimize and improve *in vitro* models, with gut-on-a-chip models being among the most promising developments. This technology aims to replicate the complex structure and functions of the gut, including the interaction between intestinal cells, microbiota, immune responses, peristaltic movements, and flow.

Recent work has been done to fill the gap in the lack of available *in vitro* models to study gut microbiome-nervous system interactions. Sedrani et al[28] were the first to publish a model called “neuroHuMiX”, which allows for the co-culture of bacterial, epithelial, and neuronal cells across microfluidic channels, separated by semi-permeable membranes. An enhanced *in vitro* model of necrotizing enterocolitis (NEC), called NEC-on-a-chip, has been developed by Frazer et al[29]. This model includes intestinal enteroids, human endothelial cells, and the microbiome of an infant with severe NEC. It is intended for mechanistic studies of NEC pathophysiology and therapy discovery. In addition, in the work of Lee et al[30], Gut Microbiome-on-a-Chip (GMoC) with great imaging capability and scalability was presented. This chip features a reproducible 3D gut epithelium derived from Caco-2 cells (μGut), mimicking key intestinal structures, functions, and cellular complexity. Depending on the bacteria type that is added (tumorigenic, such as enterotoxigenic *Bacteroides fragilis,* or beneficial, such as *Lactobacillus* spp.), authors report distinct behavior and structural changes of the μGut. Therefore, the authors suggest that the GMoC is a valuable tool for studying the roles of gut microbes in pathogenesis and developing microbe-based therapies[30]. Finally, Hayes et al[31] presented a platform to investigate the causal role of gaseous microbial metabolites within a low-gas-permeable, humanized gut microphysiological system, allowing for high-resolution analysis of host-microbe interactions. *E. coli* was engineered to regulate hydrogen sulfide levels, resulting in dose-dependent effects on host gene expression and metabolism[31] (Table 1).

MUCUS MODELS AND SCAFFOLDS

The intestinal mucus layer, produced by goblet cells, plays a significant role in shaping the composition of bacteria and protecting the gastrointestinal lining from pathogens, making it a crucial component in the host-microbiota interplay[32]. Medina and Miller[33] described a gel-like material (fluorine-assisted mucus surrogate), mimicking the double-layer architecture of native human colonic mucus that can be supplemented with various mucins to serve as a niche for microbes and be integrated with human colorectal epithelial cells to generate a multicellular *in vitro* system. Authors present this system as a simple, inexpensive alternative to stem cell-based organoid or microfluidic models that can serve as a screening platform for the high-throughput studies of host-mucus-microbe interactions[33]. Another epithelial cell-free mucus-containing system was presented by Calvigioni et al[35], where a previously described electrospun gelatin scaffold[34] was coated with mucins. Although no differences in biofilm formation resulted from mucin addition, added fecal microbiota displayed different microbial compositions in the presence of mucus[35] (Table 1).

CONCLUSIONS

In summary, the past year provided new evidence on the complex interplay between host and gut microbiota (Table 1). Various *in vitro* systems that enabled comprehensive mechanistic studies enabled significant advances in the area. In addition to the use of the conventional immortalized cell monoculture systems, 3D intestinal organoids and their polarized monolayers are rapidly emerging as more powerful tools to mimic the physiological microenvironment and assess the relationship between gut epithelium and microbiota community members. However, as these model systems are purely epithelial, efforts are being made to integrate additional immune cell types or cellular products (e.g., mucus) in various formats. As a result, organoid-derived co-cultures and more advanced gut-on-a-chip systems are emerging as indispensable tools for more accurate insights. Methodological progress in the development and usage of *in vitro* models is ongoing, and improvements to experimental systems will undoubtedly continue to fill gaps in our current knowledge in the coming year.

Conflict of Interest

All authors declare no potential conflicts of interest.

Authors' Contribution

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