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REVIEW

The role of mucus in adhesion and invasion of foodborne pathogens: challenges in current human intestinal model

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Abstract

Cell adhesion and invasion are the fundamental processes of bacterial pathogenicity that govern their probable transmission pathways. The gastrointestinal mucosa, which is lined with epithelial cells, is the primary route used by the foodborne pathogen to reach systemic organs and tissues. This mucosa is protected by a layer of continually secreted mucus, which is thought to be the initial line of defence against pathogen invasion. Studies on the adhesion and invasion ability of foodborne pathogens using Caco-2 monoculture have been comprehensively reported. This cell line, however, is classified as non-mucus-producing cells. Since the mechanism of adhesion and invasion is largely depending on the presence of mucus, the use of this cell line to study how foodborne pathogens cross the intestinal barrier has raised concerns as the establishment of the typical components that define the intestine is not established. Therefore, HT29 (low-mucus producing) and its sub-population HT29-MTX (high-mucus producing) monoculture cells have been chosen in various investigations to study the role of mucus in bacterial adhesion and invasion. However, employing monoculture as a model to study how foodborne pathogens cross the intestinal barrier faces significant challenges in mimicking the complexity of intact three-dimensional (3D) *in vivo* conditions. To address this issue, 3D co-culture models of the human intestine have been established as an alternative to the monoculture epithelial cells, allowing more accurate prediction of adhesion/invasion mechanisms. Thus, this article reviewed the role of mucus in adhesion/invasion studies of foodborne pathogens and discusses how the employment of diverse *in vitro* models impacts the properties of host-pathogen interactions.

1. Introduction

The entry of foodborne pathogens into gastrointestinal mucosa is essential for its pathogenicity, as the virulent effects of intestinal pathogens rely on their ability to colonize and invade the intestinal mucosa (Costello *et al*., 2014; El-hadad *et al*., 2016; Sharma and Kanwar, 2017; Sharma and Kanwar *et al*., 2021; Mechesso *et al*., 2021). To initiate pathogenicity, pathogens must adhere to and invade the intestinal mucosal barrier (Ribet and Cossart, 2015; Birhanu *et al*., 2018), which is protected by an overlying mucus layer that serves as the first line of defence against pathogenic microorganisms and harmful substances (Cornick *et al*., 2015; Martens *et al*., 2018; Josenhans *et al*., 2020). The foodborne pathogens appear to have developed specialized mechanisms to interact with intestinal mucus for nutrient acquisition, mucosal adhesion and traversing the mucus barrier (Dharmani *et al*., 2008; Dostal *et al*.,

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2014; Josenhans *et al*., 2020). Thus, the role of mucus in the adhesion and invasion of several foodborne pathogens such as *Salmonella enterica* serovar Typhimurium, *Listeria* spp., *Escherichia coli* and *Campylobacter jejuni* will be reviewed.

The majority of studies on the *in vitro* adhesion and invasion abilities of foodborne pathogens have been conducted using the monoculture of the Caco-2 cell line. The Caco-2 cell lines originated from human colon carcinoma, which can be differentiated into enterocyte-like cells with a microvillous structure resembling the epithelium of the small intestine (Hasbullah *et al*., 2021). Nevertheless, the Caco-2 cell line is classified as a non-mucus-producing cell. On the other hand, two other enterocytes-like cells namely HT29 and its methotrexate-treated sub-clone (HT29-MTX) are considered low-mucus and high-mucus producing cells respectively (Behrens *et al*., 2001; Laparra and Sanz, 2009; Gagnon *et al*., 2013) have been served as useful cell lines to study the role of mucus in host-pathogen interactions (Keely *et al*., 2011). The use of monocultures of epithelial cells has shortcomings that make it challenging to replicate the *in vivo* physiological environment of the intestine and fails to closely mimic the composition of the normal epithelial layer which contains a variety of cell types (Leonard *et al*., 2010; Costa and Ahluwalia, 2019; Gibb *et al*., 2021).

The intestinal epithelium is a complex environment composed of absorptive enterocytes, mucus-producing goblet cells, M-cells, as well as proliferating stem cells and Paneth cells located in intestinal crypts (Sancho *et al*., 2003; Allaire *et al*., 2018; Martens *et al*., 2018). While substantial studies on adhesion and invasion have been done on the monoculture cell line, the discovery of their mechanism in co-culture that mimics human intestinal physiology is limited. Hence, to address this issue, a 3D co-culture model based on two major phenotypes of the human intestine which are enterocytes and goblet mucus-producing cells (Caco-2/HT29-MTX) has been proposed to reconcile the complex function and barrier configuration between the apical and basolateral compartments of the intestinal *in vivo* conditions (Pereira *et al*., 2016).

Thus, this review elucidates how bacteria interact with mucus, which serves as a protective barrier in intestinal mucosa and discusses the role of mucus in adhesion/invasion studies of foodborne pathogens by presenting the current *in vitro* models that have been used to evaluate host-pathogen interactions, from basic monoculture (Caco-2, HT29 and HT29-MTX) to the more complex 3D co-culture models.

2. Colonization of foodborne pathogens on the human intestine

The mucosa surface of the intestinal epithelium acts as a frontline barrier, posing a challenge to the immune system by limiting the attachment and colonization of invading pathogens (Dharmani *et al*., 2008; Kim and Ho, 2010; Ribet and Cossart, 2015; Allaire *et al*., 2018). As the virulent effects of intestinal pathogens rely on their ability to colonize and invade the intestinal mucosa, the entry of foodborne pathogens into intestinal cells is essential for its pathogenicity, intracellular replication, spread to other tissues, and cause intestinal disease (Laparra and Sanz, 2009; Thiennimitr *et al*., 2012; Stecher *et al*., 2013; El-hadad *et al*., 2016; Mechesso *et al*., 2021). Figure 1 represents the structure of the intestinal epithelium consisting of different cell types and how enteric pathogens can cross the intestinal barrier and invade their host.

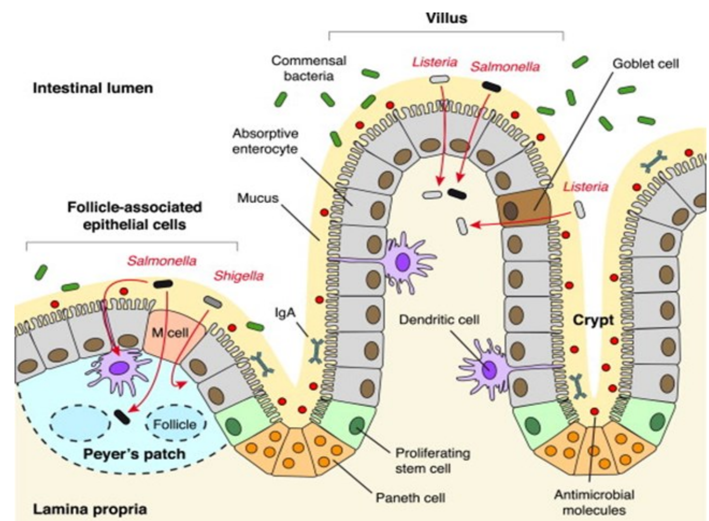

Figure 1. Structure of intestinal epithelium and the route of entry by foodborne pathogens. Source: Ribet and Cossart (2015)

The intestinal epithelium is a complex environment composed of absorptive enterocytes, mucus-producing goblet cells, M-cells, as well as proliferating stem cells and Paneth cells located in intestinal crypts. The intestinal mucus layer (represented by yellow lines) produced by mucin-secreting goblet cells protects the outer layer of intestinal epithelium against foodborne pathogenic bacteria (Dharmani *et al*., 2008; Turner, 2009; Kim and Ho, 2010; Sicard *et al*., 2017; Martens *et al*., 2018; Cai *et al*., 2020). The constituents of this mucus include glycoproteins called mucins, digestive enzymes, antimicrobial molecules and immunoglobulins (IgA) that form 'innate immune response' and aid in limiting the colonization of foodborne pathogens (Suzuki *et al*., 2004; Dharmani *et al*., 2008; Kim and Ho, 2010; Ribet and Cossart, 2015). Interestingly, despite serving as the frontline barrier, mucus-secreting cells also constitute the entry portals for foodborne pathogens by offering attachment sites (receptor for potential

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pathogens) and carbon sources that contribute essential role in their colonization (Harel *et al*., 1993; Elderman *et al*., 2017; Sicard *et al*., 2017; Martens *et al*., 2018; Cai *et al*., 2020). For example, goblet cells can be an alternative target site for *Listeria* spp. entrance (Nikitas *et al*., 2011) due to the mucin-binding protein domain of *Listeria* surface protein which allows them to adhere to mucin (Bierne *et al*., 2007; Mariscotti *et al*., 2014).

3. Human intestinal model: challenges in mimicking the *in vivo* conditions

One of the challenges in simulating the physiological environment of the human intestine is the construction of ideal biomimetic *in vitro* models that recapitulate all the essential features of the biological counterpart (Costa and Ahluwalia, 2019). Ideally, an *in vitro* model that closely resembles the human intestinal epithelium should comprise a combination of the different gastrointestinal cells. However, in most *in vitro* experimental models for understanding adhesion/invasion of foodborne pathogens, the epithelial cells of the human intestine are cultivated on flat plastic surfaces of cell culture plates as two-dimensional (2D) monocultures such as Caco-2, HT29 and HT29-MTX.

These monoculture models, however, do not fully represent the functional epithelial features and complexity of intact 3D *in vivo* intestinal physiology (Abbott, 2003; Schemeichel and Bissell, 2003; Bermudez-Brito *et al*., 2013) despite it being well known that bacterial colonization is greatly dependent on their 3D niche (Marzorati *et al*., 2011; Costello *et al*., 2014). This consideration has led to a method for bridging the gap between simple *in vitro* monoculture models and *in vivo* biological processes (Dostal *et al*., 2014; Volstatova *et al*., 2016). As an improvement, the 3D co-culture model of human intestinal cells has been developed by using the Transwell® insert (Rieux *et al*., 2007; Bermudez-Brito *et al*., 2013; Pereira *et al*., 2016; Lozoya -Agullo *et al*., 2017; Costa and Ahluwalia, 2019), as represented in Figure 2.

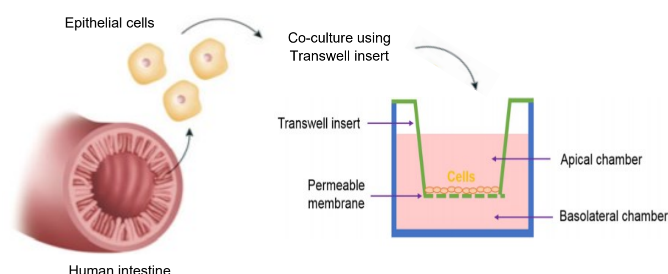

Figure 2. 3D *in vitro* co-culture model of human intestinal cells that grow on Transwell® insert. Source: Adapted from Costa and Ahluwalia (2019) and Bermudez-Brito *et al*. (2013)

The Transwell® insert represents the spatial context that separates the apical lumen from the basolateral compartment, simulating the *in vivo* intestine with an extracellular matrix for cell proliferation and differentiation (Bermudez-Brito *et al*., 2013; Pereira *et al*., 2016). Many characteristics associated with fully differentiated functional intestinal epithelium *in vivo* were improved by the 3D co-culture grown on Transwell® inserts, including apical and basolateral polarity, functional tight junctions, extracellular matrix and brush border proteins and the expression of specific transporters (Hurley *et al*., 2016; Darling *et al*., 2020). All of these important physiological features of *in vivo* intestinal epithelium were either absent or not expressed in 2D monocultures of the same cells (Zhang, 2004; Juuti -Uusitalo *et al*., 2011). Therefore, with the goal of providing an epithelial monolayer covered with mucus that better mimicked the situation *in vivo*, several studies employed the use of the Caco-2/HT29-MTX co-culture model to explore the interactions of the microbial-host interaction through adhesion/invasion will be discussed in the next section of this review.

4. Pathogenicity of foodborne pathogens: mechanism of bacterial adhesion and invasion

The intestinal adhesion and invasion of the epithelium are important stages in initiating the pathogenicity of foodborne pathogens, as the virulent effects are dependent on their ability to adhere and invade the intestinal mucosa (Krausova *et al*., 2021; Sharma and Kanwar, 2017; Costello *et al*., 2014). Despite a wide spectrum of defence systems employed by the host, foodborne pathogens used a variety of mechanisms to target and invade host cells (Ribert and Cossart, 2015). Different mechanisms have been reported to be used by foodborne pathogens to invade the intestinal mucosa: (i) direct invasion through enterocytes ii) entry through M-cells and (ii) breaches the soluble mucus layer.

The pathogenicity of *Salmonella* spp. is demonstrated by direct enterocyte invasion, which leads to the translocation past the mucosal barrier and dissemination to systemic organs (Zhang *et al*., 2014). Some of the pathogens resist antimicrobial products (Boneca *et al*., 2007; Guerry, 2007; Raffatellu *et al*., 2009) or secrete toxins that disrupt tight junctions between adjacent enterocyte cells (McGuckin *et al*., 2011), such as *Listeria* spp. (cross the junctions between goblet and enterocyte epithelial cells) (Mengaud *et al*., 1996; Ribet and Cossart, 2015) and enteropathogenic *E. coli* (Goosney *et al*., 2000). Another strategy used by foodborne pathogens to invade the intestinal mucosa by breaching the epithelial barrier through the exploitation of transepithelial transport activity of M-cells including

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S. enterica ser. Typhimurium, *Listeria monocytogenes, Shigella* and Yersinia (Vazquez-Torres and Fang, 2000; Lencer, 2001; McGuckin *et al*., 2011; Ribet and Cossart, 2015; Martens *et al*., 2018; Rey *et al*., 2020). M-cells can be identified by their loss of microvilli suggesting that they have an altered expression of cell surface mucins and no mucus is secreted in the region (Lelouard *et al*., 2001; Gibb *et al*., 2021).

The intestinal mucus contains antimicrobial molecules, secretory IgA and other effectors molecules that constitute the outermost line of the physical barrier that protects the intestinal epithelium against pathogens (Halm and Halm, 2000; Ribet and Cossart, 2015; Martens *et al*., 2018). Some bacterial pathogens have evolved mechanisms to go through the mucus layer before the establishment of colonization (Ribet and Cossart, 2015; Cai *et al*., 2020). They either produce proteases to degrade mucus (McGuckin *et al*., 2011; Sicard *et al*., 2017; Martens *et al*., 2018), or adhere directly to the mucins before the invasion of epithelia, such as *S. enterica* ser. Typhimurium, *Listeria* spp. and *E. coli* (Ensgraber and Loos, 1992; Vimal *et al*., 2000; Chessa *et al*., 2009; Sicard *et al*., 2017). However, in the study of intestinal disease and infection, the role of the mucus layer is often overlooked (Keely *et al*., 2011). Thus, the role of mucus in the adhesion and invasion of foodborne pathogens will be discussed in the next section of this review.

5. Adhesion and invasion of foodborne pathogens: the role of mucus

Several *in vitro* studies have been conducted to evaluate the role of mucus in the adhesion and invasion properties of foodborne pathogens by using monocultures of human intestinal cell lines (Table 1).

Findings by Kerneis *et al.* (1994) showed that intestinal mucus has no influence on enterotoxigenic *E. coli* (ETEC) colonization. They discovered that human ETEC binds in preference to the brush border of the enterocytic cells rather than to mucus. Meanwhile, a study by Moroni *et al.* (2006) on the adhesion and invasion pattern of *L. monocytogenes* revealed no significant differences in both Caco-2 (non-mucus producing) and HT29 (low-mucus producing) but it is of interest to note that the level of invasion was higher with HT29 cells as compared to Caco-2 cells.

On the other hand, some of the researchers conclude that the presence of mucus plays an important role as a protective component of the normal intestinal epithelium, preventing foodborne pathogens from colonizing the intestine. Keely *et al.* (2011) demonstrated a study to investigate how mucus integrity influenced host-bacterial interactions in HT29-MTX monoculture. Their findings revealed that the invasion of *S. enterica* ser. Typhimurium was significantly increased following the removal of the mucus-gel layer in HT29-MTX cells. They hypothesized that potentially invading bacteria may be physiologically hindered by the presence of mucus. However, the mucus disruption does not alter the number of adhering bacteria. Their report was attentively followed by the study in 2018 developed by Rodrigues et al. to test the adhesion and invasion of *C. jejuni* on both HT29 and HT29-MTX. They found that the presence of mucus acts as a protective barrier, attenuating the capacity of *C. jejuni* strains to adhere and invade HT29-MTX as compared to the HT29 cells. These results highlighted the importance of the mucus layer as an innate defence against intestinal mucosa and play a key role as a frontline barrier by limiting the adhesion invasion of foodborne pathogenic bacteria (McAuley *et al*., 2007; Ribet and Cossart, 2015).

However, there are several contradicting data that have been reported on the role of mucus towards the adhesion and invasion of foodborne pathogens. The presence of mucus by HT29-MTX cells, in particular, is thought to play a crucial function that aids in promoting bacterial infection since it is rich in nutrients and may ensure bacterial persistence in the host. For example, the

Table 1. Studies on the role of mucus towards adhesion and invasion of foodborne pathogens using monoculture of human intestinal cells

Monoculture	Foodborne Pathogens	Adhesion Study	Invasion Study	References
Caco-2	*Salmonella*	+	+	Gagnon *et al*. (2013)
		-	+	Li *et al*. (2019)
HT29	*Salmonella*	+	+	Gagnon *et al*. (2013)
		-	+	Li *et al*. (2019)
	Listeria	+	+	Moroni *et al*. (2006)
	E. coli	+	-	Kerneis *et al*. (1994)
	Campylobacter	+	+	Rodrigues *et al*. (2018); Alemka *et al*. (2010)
		+	-	Naughton *et al*. (2013)
HT29-MTX	*Salmonella*	+	+	Gagnon *et al*. (2013); Keely *et al*. (2011)
		-	+	Li *et al*. (2019)
	E. coli	+	-	Kerneis *et al*. (1994)
	Campylobacter	+	+	Rodrigues *et al*. (2018); Alemka *et al*. (2010)
		+	-	Naughton *et al*. (2013)

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number of *C. jejuni* colonizing HT29-MTX is significantly higher than the number colonizing their parental HT29 cell line (Naughton *et al*., 2013). They conclude that mucus gel acts as an infectious reservoir providing a steady supply of organisms that can interact with the underlying epithelium and cause disease. This finding is consistent with those of Alemka *et al*. (2010), who confirm that the overlying mucus layer on HT29-MTX cells supports *C. jejuni* reproduction and enhances their adhesion and internalization by 10-fold as compared to HT29 cells. Gagnon *et al*. (2013) found that *Salmonella* adhesion and invasion were more effective in HT29-MTX than in non- and low-mucus-producing Caco-2 or HT29 cells, respectively. They also suggested that *Salmonella* might potentially permeate the protective mucus layer and subvert the mucus to facilitate invasion. Similarly, in 2019, Li *et al*. investigated the function of MUC1 (highly expressed mucins in the stomach and intestinal tract) during the invasion of *Salmonella* to Caco-2, HT29 and HT29-MTX cell lines. According to the results, Caco-2 and HT29 had a lower level of invasion than HT29-MTX cells. They also discovered that, as compared to HT29-MTX, Caco-2 and HT29 cells express comparatively low levels of MUC1. Therefore, these findings highlight the role of mucus in promoting bacterial infection through pathogens' adhesion and invasion. As such, the HT29-MTX cells offer an ideal platform for examining the role of the mucous as a staging point for pathogenic infection at the mucosal surface (Robertson *et al*., 2000; Collado *et al*., 2005; Alemka *et al*., 2010; Keely *et al*., 2011).

6. Co-culture human intestinal model for adhesion and invasion studies

It is undoubted that one single cell line or monoculture does not accurately simulate the physiological environment of the human intestine (Hasbullah *et al*., 2021). This consideration has led to a method for bridging the gap between simple *in vitro* models and *in vivo* biological processes (Dostal *et al*., 2014; Volstatova *et al*., 2016). Consistent with the hypothesis that mucus plays a significant role in the adhesion and invasion of foodborne pathogens, the co-culture cell model consisting of two major cell phenotypes in the human intestine: absorptive enterocytes (Caco-2) and goblet mucus-producing cells (HT29-MTX) was developed to imitate *in vivo* human intestinal physiology (Pontier *et al*., 2001; Laparra and Sanz, 2009). Several researchers have used this co-culture model as a better approach to mimicking real intestinal physiology to study the adhesion/invasion of foodborne pathogens (Table 2).

Laparra and Sanz (2009) used a different *in vitro* model to test the adhesion of pathogenic bacteria (*E. coli* and *L. monocytogenes*) as well as probiotics and commensal. With the goal of providing an epithelial layer covered with mucus that better mimicked the situation *in vivo*, they used co-culture of Caco-2/HT29-MTX and compared the adhesion pattern with their respective monoculture of Caco-2 and HT29-MTX cell lines. The adhesion values of all strains tested were markedly lower in mucus-producing HT29-MTX cell cultures as compared to the values obtained with Caco-2 cell cultures. Similarly, low adhesion was detected in co-culture Caco-2/HT29-MTX than those in monocultures. These findings indicated that the mucus produced by HT29-MTX cells may cover potential membrane receptors of Caco-2 cells thus reducing bacterial adhesion.

Their results are in line with the study by Limage *et al*. (2020) that used a similar *in vitro* model of the gastrointestinal tract, the Caco-2/HT29-MTX co-culture grown on Transwell® insert. This model was employed to determine how the mucus layer was affected by the presence of *E. coli*. and commensal *Lactobacillus rhamnosus.* They discovered that mucus thickness was increased and secretion of both neutral and acidic mucins was changed following adhesion of *E. coli*. and *L. rhamnosus.* They claimed that an increase in secretion and production of mucins is related to host strategies to fight against the presence of possible pathogens. Dostal *et al*. (2014) also employed the Caco-2/HT29-MTX co-culture model grown on Transwell® insert as a physiological resemblance of *in vivo* intestine for adhesion and invasion pattern of *S. enterica* ser. Typhimurium. However, they were focused on the influence of environmental parameters such as bacterial composition and metabolite production towards bacterial adhesion and invasion.

The findings by Laparra and Sanz (2009) and Limage *et al*. (2020) are supported by the fact that the

Table 2. Caco-2/HT29-MTX co-culture model for adhesion and invasion of foodborne pathogens

No.	Description of study	References
1.	To evaluate the adhesion ability of intestinal bacteria using different *in vitro* intestinal models, and to estimate the suitability of these models	Laparra and Sanz (2009)
2.	Evaluation of the effect of mucus layer by the presence of *E. coli* and commensal *L. rhamnosus*	Limage *et al*. (2020)
3.	To investigate the impact of different iron (Fe) concentrations in the gut lumen on the modulation of adhesion and invasion pattern of *S. enterica* ser. Typhimurium.	Dostal *et al*. (2014)

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presence of mucus in the model system is significant for estimating intestinal permeability as the mucus acts as a barrier against the absorption of certain compounds (Behrens *et al*., 2001). The lack of mucus in Caco-2, on the other hand, permits easy access to the cells, leading to an overestimation of their permeability (Kleiveland, 2015). Consequently, the presence of intestinal mucus may significantly affect adherence, as observed in studies with cell lines that secrete or do not secrete mucus.

Until now, no research on the use of co-culture Caco-2 and low mucus production cells HT29 to evaluate bacterial adhesion and invasion has been reported. The Caco-2/HT29 co-culture model was primarily utilized to investigate the permeability of the nanogels and several peptide drugs in the intestine (Antunes *et al*., 2013; Xavier *et al*., 2019). Moreover, the use of simple monoculture Caco-2 cells does not contemplate the important factors human intestine that influence the functionality of enterocytes such as the mucus layer or the interactions between the epithelium and the stroma (Li *et al*., 2013). As a result, the co-culture of Caco-2/HT29-MTX covered with mucus might be deemed the most acceptable *in vitro* model of human intestinal epithelium for understanding bacterial adhesion and invasion, and the results produced using this co-culture model will closely mimic the human gut.

7. Conclusion

The Caco-2 cell line monoculture has long been the most widely used and approved *in vitro* intestinal cell model for studying the adherence and invasion of a foodborne pathogen. The presence of mucus, an important protective component of the normal intestinal epithelium, is, nonetheless, crucial to the adhesion and invasion mechanism. As a result, compared to Caco-2 and HT29 cell models that exude no or little mucus, the HT29-MTX cell model may be better for evaluating *in vitro* bacterial adhesion and invasion. Due to the presence of both absorptive enterocytes and goblet mucus-producing cells, which are the most important features representing human intestinal mucosa, the co-culture of Caco-2 cells with the mucus-producing HT29-MTX cell line has been referred to as a more predictable experimental cell model than monoculture alone. Data from this review suggest that significant progress has been made towards the development of 3D co-culture of Caco-2/HT29-MTX cell model grown on the Transwell® insert, with better physiologically relevant characteristics of mucus layer formation, herein creating an excellent and reliable *in vitro* method for characterizing cells–pathogens interactions via adhesion/invasion study. This advance led to a greater increase in our understanding of host-pathogen interaction in a more complex environment through the 3D mucosa model, diminishing the use of simple 2D monoculture. Utilising multidisciplinary knowledge from biology and engineering is likely to become important in enhancing the current methodologies for adhesion and invasion study due to the multifaceted nature challenges of co-culture systems.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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