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Intestinal mucus: the unsung hero in the battle against viral gastroenteritis

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Abstract
Intestinal mucus plays a crucial role in defending against enteric infections by protecting the vulnerable intestinal epithelial cells both physically and through its various constituents. Despite this, numerous gastroenteritis-causing viruses, such as rotavirus, coronavirus, adenovirus, astrovirus, calicivirus, and enterovirus, continue to pose significant threats to humans and animals. While several studies have examined the interactions between these viruses and intestinal mucus, significant gaps remain in understanding the full protective potential of intestinal mucus against these pathogens. This review aims to elucidate the protective role of intestinal mucus in viral gastroenteritis. It begins with a comprehensive literature overview of (i) intestinal mucus, (ii) enteric viruses of medical and veterinary importance, and (iii) the known interactions between various enteric viruses and intestinal mucus. Following this, a case study is presented to highlight the age-dependent blocking effect of porcine intestinal mucus against transmissible gastroenteritis virus, a porcine coronavirus. Finally, the review discusses future investigation directions to further explore the potential of intestinal mucus as a defense mechanism against viral gastroenteritis to stimulate further research in this dynamic and critical area.

Keywords Intestinal mucus, Viral gastroenteritis, Virus-mucus interaction, Mucosal immunology, Virus-blocking

Intestinal mucus

Before going into the details of interaction of intestinal mucus with enteric viruses, it is imperative to get an in-depth idea of its structure, composition, and physiochemical properties. Mucus is a dilute, aqueous, and viscoelastic secretion, mainly composed of water (90–95%), proteins (~5%), lipids (1–2%), and electrolytes, forming a colloidal solution [1]. Small intestinal mucus forms a single, loose, and easily removable layer over enterocytes, measuring approximately 25.9 ± 11.8 µm in the duodenum and 31.0 ± 15.7 µm in the ileum of pigs. In the colon, this loose layer contains bacteria and digesta, while a second, firmly attached mucus layer (35.1 ± 16.0 µm in the descending colon of pigs) lies close to the epithelial lining, free of bacteria and food particles [2–4]. This sterile layer is essential for protecting the colon epithelium from the large population of microflora present in the colon [1–2]. In rats, the estimated mucus thickness is about 28.8 ± 25.5 µm in the duodenum, 93.3 ± 59.4 µm in the jejunum, and 41.3 ± 16.5 µm in the distal colon [5]. In contrast, in mice, the mucus thickness is approximately 20 µm in the small intestine and around 116 µm in the colon [6]. The variations might be due to species, breed, or age differences as well as processing methods. Relevant data in humans is limited due to difficulty in sample availability and needs further exploration. Mucin (MUC) glycoproteins, the main building blocks of mucus, are synthesized and secreted by specialized intestinal

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epithelial single-cell glands called goblet cells [7]. They form a net-like sheet covering the villi, making micropores which create gaps between adjacent mucin sheets, allowing the passage of larger particles, including bacteria [8]. About 80–85% of the mucin mass is composed of *O*-glycans, which after binding with water, give mucus its gel-like appearance [9]. The central protein domain of mucins usually contain large numbers of proline, threonine, and serine (PTS) domains, which after linking with glycans, create mucin domains [10]. The three-dimensional surface made by their terminal glycans can interact with cells or microorganisms [9]. They are generally categorized as transmembrane mucins and secreted mucins [11]. Commonly expressed mucins in the (gastro)intestinal tract are shown in Table 1.

Mucins are highly glycosylated glycoproteins. In the PTS domains, threonine and serine (hydroxy amino acids) are *O*-glycosylated, forming long, stiff mucin domains [23]. In MUC2, the mucin domain of a single monomer can contain up to 1600 *O*-glycans and 30 *N*-glycans, resulting in a massive glycan array of 3300 sugar residues, which offer interaction sites for commensal bacteria and invading pathogens, including viruses [24]. This glycan array can terminate in a variety of sugar molecules, of which molecules like sialic acids and histo-blood group antigens (HBGAs) express on the enterocyte surface and have been recognized as attachment sites for rotaviruses [25]. This attachment can potentially facilitate the entry of viruses into host cells, particularly with tethered mucins located at the cell surface. Conversely, soluble mucins may utilize these glycan attachment sites to trap viruses, aiding in their removal from the intestinal tract through mucus transport. Studies on transmissible gastroenteritis virus (TGEV) has shown its affinity to different sialic acids, with highest for 5-N-glycolylneuraminic acid (Neu5Gc) followed by 5-N-acetylneuraminic acid (Neu5Ac) and 5-N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2) [26, 27]. All three sugars have been described as receptors or receptor binding co-factors for different coronaviruses [26]. Thus, it can be inferred that mucin glycans, particularly related to sialic acid moieties, offer much potential in understanding the interaction and pathogenesis of enteric viruses. Apart from mucins, other proteins modulating the host's immune response such as Immunoglobulin A (IgA), lysozymes, defensins, deleted in malignant brain tumors 1 (DMBT1), regenerating islet-derived protein 3 alpha (REG3A), calcium-activated chloride channel regulator 1 (CLCA1), IgG Fc-binding protein (FCGBP), anterior gradient protein 2 homolog (AGR2), zymogen granule membrane protein 16 (ZG16), kallikrein 1 (KLK1), and trefoil factor 3 (TFF3), are also present in the intestinal mucus [9, 28–31].

Other mucus components like lipids constitute 1–2% of mucus and mainly include phospholipids like phosphatidyl choline and phosphatidyl glycerol, but in some pathological conditions, cholesterol and free and acylated fatty acids can be observed [32]. Neutral lipids interact with mucins, affecting their hydrophobicity, while charged lipids affect the wettability of the mucus layer [33]. Furthermore, lipids contribute towards surface tension, lubrication, rheological properties, and prevention of evaporation of aqueous contents of mucus [32]. Lipids from dietary sources alter the microbial transport as documented in reduced motility of *E. coli* in intestinal mucus with high fat diet [34]. The percentage of minerals and electrolytes like magnesium and calcium, sodium and potassium chloride, sodium bicarbonate, and phosphates in intestinal mucus is highly variable and defined by the underlying secretory epithelium [32]. These mainly contribute to controlling mucus viscosity and hydration, as evident by loss of mucus gel structure by increased magnesium and calcium [35], or reduced mucus viscosity by increased concentration of sodium or potassium [32].

Table 1 Commonly expressed transmembrane and secreted mucins in the (gastro)intestinal tract

Type of Mucin	Mucin	Characteristics	Function
Transmembrane (tethered) [9, 12–17]	MUC3-4, MUC12-13, MUC17 (constitutive), MUC1, MUC16 (upregulated during infections and cancers)	Type 1 glycoproteins with a single transmembrane domain; N-terminal at the apical surface of enterocytes	Cell signaling, creating glycocalyx, protecting enterocyte cell membrane
Secreted (gel-forming) [9, 18–22]	MUC2 (principal in intestines), MUC6 (gastric and duodenal glands), MUC5B (low levels in colon), MUC5AC (upregulated during intestinal nematode infections)	Cysteine-rich N- and C-terminal domains mediating oligomerization	Forming the skeleton of the intestinal mucus layer

MUC: mucin

Viral gastroenteritis

Acute viral gastroenteritis is one of the most common cause of morbidity and mortality in humans and animals worldwide [36, 37]. Children under five years of age and young suckling animals are particularly prone [38, 39]. The extremely high mortality associated with gastroenteritis has been estimated to be 3–5 million cases per year in humans, the majority of which occur in developing countries [38]. In the developed world, it is associated with high morbidity and high incidence of hospitalization [40]. Similarly, it has a huge economic impact on the livestock industry, as observed in dairy cattle in terms of high mortality, loss of production, and cost of medication and vaccinations [41]. Below is a concise summary

highlighting the key structural features, pathogenesis, and immune responses associated with major gastroenteritis-causing viruses in both humans and animals:

Rotaviruses

Rotavirus (RV) is a genus of multilayered non-enveloped viruses of about 100 nm diameter in the family *Reoviridae* [42, 43]. The triple-layered icosahedral capsid encloses a genome of 11 segments of double-stranded RNA (dsRNA), each coding for one or two viral proteins [44]. The species are based on serogroups A-J and putative species RVK and RVL are already reported [45]. The outer layer of the capsid contains VP7 and VP4 proteins that are important for viral attachment, entry, and antigenicity while also determining rotavirus serotype and strain [46]. These proteins are also called G (for glycoprotein) and P (for protease-sensitive) types, respectively [47]. Sialic acids, integrins and hsc70 have been reported as functional receptors for many RVs [48, 49]. Additionally, our lab's work on primary porcine enterocytes co-cultured with porcine myofibroblasts infected with porcine rotavirus hinted towards the usage of a basolateral intercellular receptor [50]. There are at least 27 G types, and 37 P types of rotavirus identified so far, though only a few of them are common in humans and animals [51]. They also go under genetic reassortment and carry a zoonotic potential [52]. However, genome characterization of ancient and recent Belgian pig RVAs from our lab has indicated a different evolutionary path with human Wa-like RVAs [53], suggesting a better adaptation of pig RVAs to porcine enterocytes and less chances of spread to human populations. In humans, the diseases severity of rotavirus infections is higher than other enteric pathogens [54]. Inflammation of the stomach and intestines leading to diarrhea, vomiting, fever, and dehydration are the main clinical signs [55]. Neonates are generally less affected probably due to protection from maternal antibodies, yet infections are more severe in young children and animals [56]. Work on porcine rotavirus from our lab in Belgian pig farms has identified RVA with heterogenous VP7/VP4 genotype combinations [48], subclinical RVA [57], along with RVA and RVC [58]. A detail of common rotavirus species along with common genotypes with respect to their host is provided in Fig. 1 [59–61].

Coronaviruses

Coronaviruses (CoV) are large, enveloped viruses of the family *Coronaviridae* containing positive-sense single-stranded RNA (ssRNA) enclosed within the nucleocapsid protein of around 30 kbps size [62]. They have four main structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N), arranged in a specific pattern that gives the virus its characteristic crown-shaped (corona) appearance [63]. Certain members of the *Betacoronavirus* genus, particularly those in lineage A, possess an additional hemagglutinin-esterase (HE) structural protein, exhibiting lectin-like activity by binding to sialic acid [64], making it crucial for studying its interactions with mucus. The S protein has two subunits: a receptor-binding domain (RBD, S1) and the membrane-fusion domain (S2) [65], making it a target of many vaccines and therapeutics against coronaviruses [66]. Receptor usage depends on virus type. For example, SARS-CoV-2 uses angiotensin converting enzyme 2 (ACE2) [67] while porcine coronaviruses like transmissible gastroenteritis virus (TGEV) mainly use aminopeptidase N (APN) [68]. Another study from our lab reported a two-to-seven-fold higher infectivity of TGEV Miller in APN positive cells than in APN negative cells, but TGEV Purdue replicated better in APN negative cells [69]. The same study reported that terminal sialic acids are not determinants of TGEV infection. This shows that receptor usage of coronaviruses is highly variable. Although coronaviruses are mainly associated with respiratory infections in humans, they are known causative agents of neonatal diarrhea in many animals [70], as shown in Fig. 1. In humans, studies on severe acute respiratory syndrome-CoV-2 have also identified the presence of the virus in fecal samples, suggesting that it may be capable of causing gastrointestinal infections as well [71]. In addition, there are several other coronaviruses that have been detected during gastrointestinal infections in children, including 229E, OC43, HKU1, NL63 [72]. Moreover, research on the replication of swine acute diarrhea syndrome coronavirus in primary human cells indicates that humans may be susceptible to this virus, highlighting a potential zoonotic threat [73]. Similarly, the zoonotic nature of porcine delta coronavirus (PDCoV) has also recently demonstrated with detection in plasma samples of hospitalized children in Haiti [74]. Animal coronaviruses cause extensive necrosis of mature jejunal and ileal enterocytes within 24 h of infection, reducing enzymatic activity (mainly alkaline phosphatase and lactase) and disrupting digestion and electrolyte imbalance, leading to fluid deposition in the intestinal lumen, causing acute malabsorptive diarrhea [75]. The resulting dehydration and loss of extravascular proteins is fatal and could lead to metabolic acidosis, increased K^+ levels, and eventually cardiac arrest [76]. Main symptoms include severe diarrhea, anorexia, emaciation, and dehydration [77]. Infection is more severe in newborn animals with mortality rates reaching 100% in the absence of lactogenic immunity [78].

Enteric adenoviruses

Adenoviruses are non-enveloped, icosahedral viruses of the family *Adenoviridae*, of 70–90 nm diameter, and have a linear dsDNA genome of approximately 26–48 kbps

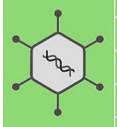
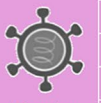
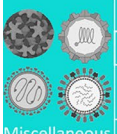
Virus type	Common virus species	Host
 Rotavirus	RVA (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8])	Human
	RVA, RVB, RVC (G6P[5], G8P[1], G10P[11])	Cattle
	RVA, RVB, RVC (G4P[6], G5P[7], G9P[13])	Pig
	RVB (G6P[14], G8P[15])	Sheep
	RVA (G3P[12], G14P[12])	Horse
	RVA (G3P[3], G6P[3])	Dog
	RVA (G3P[9], G6P[9])	Cat
	RVD, RVF, RVG (G1P[1], G2P[2])	Poultry
 Coronavirus	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, β-CoV)	Human
	Porcine epidemic diarrhea virus (PEDV, α-CoV)	Pigs
	Transmissible gastroenteritis virus (TGEV, α-CoV)	
	Severe acute diarrheal syndrome (SADS-CoV, α-CoV)	
	Porcine hemagglutinating encephalomyelitis virus (PHEV, β-CoV)	
	Porcine delta coronavirus (PDCoV, δ-CoV)	
	Bovine coronavirus (BCoV, β-CoV 1)	Cattle Goat Sheep
	Feline enteric coronavirus (FECV, α-CoV 1)	Cat
	Canine enteric coronavirus (CECoV, α-CoV 1)	Dog
	Equine coronavirus (ECoV, β-CoV 1)	Horse
	Infectious bronchitis virus (IBV, γ-CoV)	Poultry
	Ferret enteric coronavirus (FRECV, α-CoV)	Ferret
 Adenovirus	Human adenovirus (HAdV, *Mastadenovirus*)	Human
	Porcine adenovirus (PAdV-1, PAdV-3, PAdV-4, *Mastadenovirus*)	Pig
	Bovine adenovirus (BAdV, *Mastadenovirus* and *Atadenovirus*)	Cattle
	Goat adenovirus (GAdV, *Mastadenovirus* and *Atadenovirus*)	Goat
	Ovine adenovirus (OAdV, *Mastadenovirus* and *Atadenovirus*)	Sheep
	Canine adenovirus 2 (CAdV-2, *Mastadenovirus*)	Dog
	Equine adenovirus 2 (EAdV-2, *Mastadenovirus*)	Horse
	Cervine adenovirus (*Odocoileus adenovirus-1*, OdAdV-1)	Deer
 Astrovirus	Human astroviruses 4 and 8 (*Mamastrovirus 1*)	Human
	MLB-1 (*Mamastrovirus 6*)	
	VA1/HMO-C (*Mamastrovirus 9*)	
	Ovine astroviruses (*Mamastrovirus*)	Sheep
	Bovine astroviruses (*Mamastrovirus*)	Cattle
	Feline astroviruses (*Mamastrovirus*)	Cat
	Porcine astroviruses (*Mamastrovirus*)	Pig
	Turkey astroviruses 1 and 2 (*Avastrovirus*)	Turkey
 Miscellaneous	Human Norovirus GI and GII	Human
	Human Sapovirus (HuSaV)	
	Poliovirus (Enterovirus C)	
	Human enterovirus A, B, C	
	Bovine enterovirus A	Cattle
	Avian influenza virus (H1N1, H5N1, H7N1, H9N2)	Human Chicken
	Morbillivirus caprinae Ovine rinderpest, peste des petits ruminants (PPR)	Sheep Goat
	Canine distemper virus (footpad disease)	Dog
	Newcastle disease virus	Birds

Fig. 1 A comprehensive list of common gastroenteritis-causing viruses with details of the virus species, type, diseases and host species.

[79]. The genome is packaged into a protein capsid that consists of three main structural components: (1) penton base, a five-fold symmetric protein structure that forms the vertex and is responsible for virus attachment to the host cell; (2) hexon, the capsid protein forming the bulk of the virus particle; (3) fiber, a trimeric protein binds to specific receptors on the host cell surface [80]. The genome is bound to histone-like proteins known as protamines [81]. They are highly regarded for their use in vector-based vaccines due to their ease of genetic manipulation, inherent stability, and wide tissue tropism [82]. Enteric adenoviruses cause gastrointestinal infections in both humans and animals. Among 111 types of human adenovirus (HAdV), enteric types like F40/41 are significant acute gastroenteritis agents in children [83]. Enterocolitis-like symptoms have been associated with different adenovirus species isolated from various domestic and wild animals as well, as shown in Fig. 1 [84, 85].

Astroviruses

Astroviruses are small, non-enveloped viruses of the family *Astroviridae* containing a single-stranded RNA genome of approximately 6.8 to 7.9 kbps [86]. Common to most RNA viruses, their genomes are prone to a high mutation and recombination rate, resulting in species that can infect a wide variety of hosts [87, 88]. They are a common cause of gastroenteritis in human children and young animals all over the world [89, 90]. The symptoms include diarrhea, nausea, vomiting, abdominal cramps, low-grade fever and malaise [91]. They mainly infect the intestinal goblet cells, leading to increased mucus production and electrolyte imbalance [92]. Some common astroviruses infecting human and different animal species are described in Fig. 1 [84, 90].

Miscellaneous viruses

There are several other viruses that can cause gastrointestinal infections in humans and animals, including:

1. Caliciviruses: two genera, Norovirus (also called Norwalk virus) and Sapovirus are known to cause gastroenteritis in humans. Norovirus is a small non-enveloped virus [93] and is common in settings such as schools and nursing homes [94]. Sapovirus gastroenteritis is usually associated with mild, self-limited illness, but can cause more severe disease in immunocompromised individuals [95]. Symptoms include diarrhea, vomiting, abdominal cramps, and fever [96, 97].
2. Enterovirus: a genus that forms the largest group in the ever-expanding *Picornaviridae* family contains important enteric viruses, including poliovirus [98]. These may cause a whole range of illnesses, including gastrointestinal infections [99].
3. Avian Influenza Virus (AIV): primarily a respiratory virus, it has also been observed to replicate in the intestinal cells of chicken (H5N1 [100], H9N2 [101], H7N1 and H1N1 [102]) and humans (H9N2 [103]).
4. Paramyxoviruses: mostly associated with respiratory diseases in humans, however some members of this group are known to cause gastroenteritis-like disease in domestic animals and birds with symptoms ranging from mild to severe diarrhea [104]. A list of common miscellaneous viruses causing gastroenteritis in humans, animals and birds [96, 105–107] is also provided in Fig. 1.

Interaction of enteric viruses with intestinal mucus: a literature review

The interaction between viral particles and mucus remains a largely unexplored field. Biological mucus is a dynamic fluid due to the elasticity provided by gel-forming mucins like MUC2 [108]. The viral transport through this dynamic layer is strongly related to the particle-to-pore size ratio and is dependent on: (1) passive diffusion when particle is smaller than pore size; (2) active transport by manipulation of mucus elasticity when particle is larger than pore size; and (3) a combination of mucus elasticity and viscosity (microscopic rheology) when both particle and pore size are similar [108]. These interactions are relevant when biochemical factors are not considered; incorporating them will undoubtedly introduce additional complexity. Hence, the third condition is more interesting as most enteric viruses have comparable sizes to average mucus pore size (~50–200 nm) [109], and biochemical interactions between these viruses and mucosal components might be more prominent. As particle size increases, their diffusion rates decrease. When particles reach approximately 500 nm to 1 μm in diameter, the rheological properties of the mucus overcome their free diffusion [105]. Furthermore, mucins and their glycans, as previously mentioned, serve as significant interaction sites for sugar-recognizing enteric viruses such as rotavirus and coronavirus. For research on mucus-virus interactions, both in vivo and in vitro models have been used. However, in vivo studies are mostly based on dynamic mucus while the in vitro studies on static mucus, which often leads to inconsistent results. Table 2 presents an in-depth review of the interactions between enteric viruses and intestinal mucus, drawing from various research studies.

This suggests that various enteric viruses interact with intestinal mucus and mucins like MUC2, providing significant potential for investigating protective or blocking effects of intestinal mucus against these viruses. The following case study exemplifies how this interaction can be explored to assess the blocking effect of intestinal mucus on enteric viruses of medical and veterinary importance.

Table 2 Interaction of enteric viruses with intestinal mucus

Virus	Model	Interaction	Reference
Rhesus rotavirus (RRV)	In vitro and In vivo mouse	Virus infection decreases in the presence of mucin isolated from human milk	[110]
	In vivo mouse	↑*Bacteroides* and *Akkermansia* populations which have mucin-digesting properties, leading to ↑ RRV virulence	[111]
	In vitro	Suckling intestinal mucins neutralize RRV more effectively than adult mucins	[112]
	In vivo mouse	↑ mucin-digesting bacteria ↓ *Lactobacillus* species ↑ rotavirus virulence	[111]
		Suckling intestinal mucins neutralized RRV more effectively than adult mucins	[112]
Epizootic Diarrhea of Infant Mice (EDIM) mouse rotavirus strain		↑ MUC2 mRNA levels Potent anti-rotaviral effect of mucin isolated from 4dpi infected mice	[113]
Human rotavirus	In vitro	Possible binding of P[19] with mucin cores 2, 4, and 6	[114]
Human P[19] and P[II] genogroup (P[6], P[8], P[4])		Possible interaction of P[II] genogroup RV VP8*s with mucin core 2	[115]
RVA/Human-wt/IND/mcs60/2011/G3P		Strong P[10] binding with mucin core 2 and weak binding to mucin core 4	[116]
Simian rotavirus	In vivo mouse	Rotavirus binds to sialomucins	[117]
SARS-CoV-2	In vivo monkey	↓ Ki67 and mucin-containing goblet cells in GIT with intragastric inoculation	[118]
TGEV and PEDV	In vitro	Attenuated infection in the presence of mucus layer derived from porcine intestinal organoid air–liquid interface monolayer	[119]
PEDV	In vivo pig	Acidic mucins in PEDV-infected pigs 2dpi ↓ Goblet cells in PEDV infected pigs 1-5dpi (nursery) and 3-5dpi (weaned)	[120, 121]
	In vitro and In vivo pig	Antiviral activity of MUC2 and mucus-derived Calpain-1 on Vero E6 cells Oral administration of Calpain-1 in piglets provides resistance to infection	[122]
Feline enteric coronavirus serotype 1	In vitro	Treatment of feline intestinal epithelial cell cultures with bovine submaxillary mucin inhibits subsequent viral infection	[123]
IBV Mass-41 and IBDV serotype 1	In vitro and In vivo chicken	Calcium binding protein 1 (CALB1) derived from ileal mucus significantly suppresses the replication of both viruses	[124]
Human adenovirus (HuAdV) 5p	In vitro	Preferential infection of goblet cells in human enteroids and potent neutralization by the enteric human alpha-defensin HD5	[125]
Human norovirus GII.10 virus-like particles		Antiviral activity of porcine gastric mucin measured by ELISA in terms of IC_{50} and OD reduction	[126, 127]
Enterovirus 71 (EV71)		↓ expression of goblet cell–derived mucins	[128]
Murine astrovirus (fecal isolated)	In vivo mouse	Active infection in small intestinal goblet cells, ↑ mucus-associated bacteria, ↑ *E. coli* colonization resistance	[129]
Human astrovirus 1 and 8	In vitro	↑ virus infectivity on Caco-2 cells with ↑ dose of porcine stomach mucin	[130]
AIV H9N2	In vivo chicken	↓ mRNA expression of MUC2 at 5dpi	[100]

↑: increased/higher; ↓: decreased/lower, dpi: days post-infection; PEDV: porcine epidemic diarrhea virus; TGEV: transmissible gastroenteritis virus; IBV: Infectious Bronchitis virus; IBDV: Infectious Bursal Disease virus; IC_{50}: Half-maximal inhibitory concentration; OD: optical density; AIV: Avian influenza virus; MUC: mucin

Case study: age-dependent protective role of porcine ex vivo intestinal mucus against transmissible gastroenteritis virus (TGEV)

Now that we have an idea on how different enteric viruses interact with intestinal mucus, let us dive into a case study where we investigated the age-dependent protective effect of porcine ex vivo intestinal mucus against TGEV infection. This virus infects enterocytes of small intestines in pigs of all ages [131]. The clinical signs, including diarrhea, vomiting, dehydration, and high mortality, are very severe in young piglets during their first days of life. The mortality rate in 1 to 3-day-old piglets without lactogenic immunity can reach 70–100% [132]. The clinical signs become less pronounced with increasing age. Studies on TGEV titer in tissues of different ages of pigs suggest a higher infection rate in 3-day-old pigs

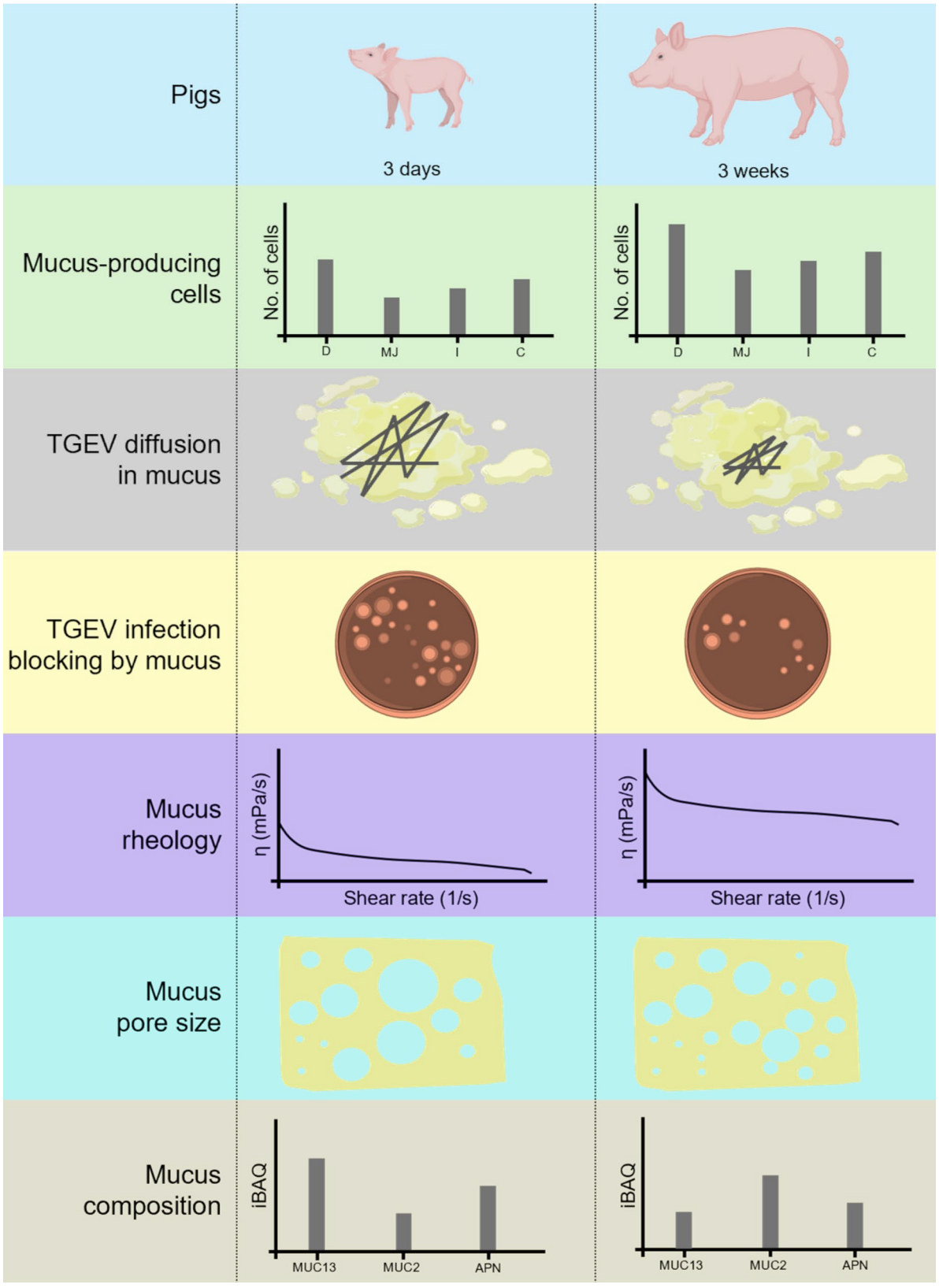


Fig. 2 (See legend on next page.)

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Fig. 2 Schematic representation of the differences in 3-day and 3-week mucus from our studies. **Mucus-producing cells**: cumulative number of mucus-producing cells in different intestinal regions (D = duodenum, MJ = mid-jejunum, I = Ileum, C = colon) is schematically represented, showing a higher number in 3-week intestines. **TGEV diffusion in mucus**: diffusion in 3-day mucus was higher as compared to 3-week mucus. **TGEV infection blocking by mucus**: a smaller number of TGEV-infected cells were observed in the presence of 3-week mucus as compared to 3-day mucus. **Mucus rheology**: 3-week mucus exhibited more viscosity (η) with an increasing shear rate as compared to the 3-day mucus. **Mucus pore size**: pore size was more variable and larger in 3-day mucus. **Mucus composition**: Intensity-based absolute quantitation (iBAQ) of MUC13, MUC2, and APN is schematically represented, which revealed that a higher expression of MUC13 and APN was observed in 3-day mucus while MUC2 was significantly higher in 3-week mucus

than 3-week-old pigs 3–4 days post-infection [133, 134]. We first thought that this age-dependent loss of susceptibility could be due to the higher expression of coronavirus receptors in intestines of young piglets. However, upon investigation, a clear correlation was not found [135]. We then investigated this correlation in terms of the expression of mucus-producing cells from two different age groups of pigs (3 days and 3 weeks) in the same study. It was concluded that the number of mucus-producing cells increased with age and may play an essential role in protecting enteric mucosae against intestinal viruses [135]. Thus, we carried further studies to explore the age-dependent, anti-TGEV protective effect of intestinal mucus along with its physiochemical properties from 3-day-old and 3-week-old pigs [109]. Figure 2 outlines these findings in a schematic manner, showing key differences between newborn (3-day-old) and peri-weaning (> 3-week-old) pigs.

The mean percentage of mucus producing cells from the duodenum to the colon increased with age with the highest mean percentage observed in the Brunner's glands of the duodenum [135]. This was checked on both paraffin embedded and cryopreserved tissues in two different settings and the results corresponded to each other. Thus, ex vivo intestinal mucus from the two age groups was collected using a previously described method [136]. Using single particle tracking (SPT), it was shown that TGEV moves more freely in 3-day mucus as compared to 3-week mucus measured in terms of diffusion coefficient calculated by means of multiple trajectories over a short period of 5 s [109]. A diffusion pattern was analyzed using an in-house system over longer periods (10 min and 30 min) and again showed that TGEV diffused significantly better in 3-day mucus than in 3-week mucus [109]. In the same study, it was demonstrated that 3-week mucus has a significant TGEV-blocking effect on susceptible swine testicular (ST) cells as compared to the 3-day mucus. Next, the physicochemical properties of the ex vivo mucus from both age groups was examined to understand the age-dependent protective effect. Using a rheometer, we showed that 3-week mucus exhibited less shear thinning (higher viscosity) as compared to 3-day mucus [137]. In the same study, we also measured the pore-size of mucus using atomic force microscopy (AFM) which ranged between 10 and 350 nm in 3-day mucus and from around 8 to 240 nm in 3-week mucus. The average pore size for 3-day mucus was 234.56 ± 129.5 nm in diameter, while that of 3-week mucus was 152.60 ± 94.4 nm. In 3-day mucus, more than 80% of the pores were larger than the average diameter of TGEV particles (~ 80–120 nm), while in 3-week mucus, about only 50% pores were larger than the virus diameter. And finally, the proteomic profile of the two mucus samples showed that MUC2, the main mucin of intestinal mucus, was more prevalent in 3-week mucus which could play a role in blocking the viral infection in older pigs, as mucins have been shown to inhibit coronavirus infection in a glycan-dependent manner [138]. Interestingly, MUC13 was significantly more expressed in 3-day mucus. This transmembrane mucin is highly expressed on the apical surface of enterocytes, and its role in negatively regulating the tight junction proteins and intestinal epithelial barrier integrity through protein kinase C has been recently identified [139]. This can also explain the increased viral susceptibility of 3-day-old pigs as epithelial integrity is decreased because of MUC13 activity. APN, the main receptor for TGEV/PRCV [140], had a higher expression in 3-day mucus. This soluble APN in mucus may drive TGEV towards susceptible epithelial cells. When taken together, all the data shows that there is an age-dependent protective effect of porcine ex vivo intestinal mucus against TGEV.

This case study exemplifies the integration of fundamental techniques such as histology with in-house developed methods, established techniques like SPT and AFM, and omics analysis to produce valuable baseline data. It also highlights the significant potential for further investigation into the protective role of intestinal mucus against viral gastroenteritis.

Future research pathways to explore intestinal mucus as a defense against viral gastroenteritis

With our enhanced understanding of how intestinal mucus interacts with enteric viruses, the next section will explore the potential applications of this knowledge in future research.

Quantifying mucus-producing cells in the intestine of a particular species

The number of mucus-producing cells along the intestinal length can give a good indication of mucus production in a particular species, age-group or individual [141]. We demonstrated that the number of mucus-producing cells per total epithelial cells increased with pig's age

along the whole intestinal length, suggesting more mucus production per unit of area in older pigs [135]. This could provide an explanation why younger piglets are more prone to severe infections and high mortality. As the fixation process during mucus staining strongly impacts the preservation of mucus in histological sections [142], traditional PAS staining on paraffin sections and a novel method of fixing cryosections on the positively charged Blotting-Nylon 66 membranes could be used [135]. The results corresponded to each other. This shows that relatively simple staining techniques can still be efficient in providing valuable data related to virology. Furthermore, region-specific changes in the intestines can also provide significant data related to enteric viral infections. The Brunner's glands found in the duodenum produce a large amount of mucus as observed in our study [135]. Mucus produced from these merocrine glands is more alkaline to counteract the acidic content coming from the stomach [143]. This could explain why the duodenum is relatively less infected by enteric viruses as compared to jejunum and ileum. A study using in-situ hybridization for the detection and localization of PEDV found strong signals in villus enterocytes of jejunum and ileum of all ten pigs, while duodenum was positive in only one pig [144].

Thus, quantifying intestinal mucus or mucus-producing cells with respect to the intestinal region, host species or age holds significant translational value across various medical fields. Measuring mucus levels can enhance the diagnosis and monitoring of conditions like inflammatory bowel disease (IBD) and ulcerative colitis, as variations in mucus production may indicate disease progression or treatment response [145]. Understanding individual differences in mucus production can help tailor treatments for gastrointestinal diseases, offering specific therapeutic approaches based on mucus levels [146]. Insights into mucus dynamics can guide the creation of new drugs, allowing researchers to design medications that modulate mucus production to improve patient outcomes [32, 146]. Additionally, studying mucus interactions with gut microbiome can lead to the development of probiotics or other interventions that support overall health [147]. As a protective barrier for the gut lining, mucus quantification can help researchers understand and address barrier dysfunctions in diseases, potentially preventing infections and inflammation.

The potential role of mucus composition changes during infections

Mucus composition is mostly explored by performing a proteomic profile consisting of mucins, enzymes, immunoglobulins, and non-mucin proteins including the immunomodulatory elements like antimicrobial peptides (AMPs) [148]. However, apart from proteins, intestinal mucus is a blend of lipids, electrolytes, and water [9]. In a study from our lab, a label-free proteomic analysis of the ex vivo intestinal mucus from 3-day-old and 3-week-old pigs revealed that around 2.75% proteomic profile was unique between the age groups [109]. There is a variety of non-mucin proteins that are in play during different infectious diseases. Secretory IgA can bind to certain bacteria forming immune complexes that promote phagocytosis [149]. ZG16 is known to combine with the peptidoglycans found in the gram-positive bacterial cell wall, forming large aggregates that cannot cross the mucus layer [150]. Receptor proteins like NLRP6 are activated during certain viral and gram-positive bacterial infections [151]. AMPs like defensins and cathelicidins possess antibacterial and antiviral properties that are mainly released into the intestinal lumen by the Paneth cells (PCs) [152–154]. Thus, characterization of these molecules in mucus samples from different ages of host species can provide a better overview of the onset, progression, and severity of enteric diseases, especially in newborn humans and suckling and naïve animals.

Non-proteinaceous parameters of intestinal mucus like the yield, pH, water content, sugars, lipids, and metabolites also considerably affect the mucus composition. Mucus pH affects virus stability as evidenced by the high antiviral activity of cervicovaginal mucus against human immunodeficiency virus 1 (HIV-1) at acidic pH [155]. Lipid profile variation in different sputum samples affects influenza and rhinovirus infection [156]. Mucus electrolytes alter mucus properties as divalent cations (Mg^{2+}, Ca^{2+}) are known to collapse the mucus gel structure while monovalent cations (Na^{+}, K^{+}) are known to reduce mucus viscosity [32], which in turn can affect virus tropism and infection. Thus, these non-proteinaceous intestinal mucosal components offer much room for future investigation in relation to viral gastroenteritis. The absolute mucus yield can be measured as the weight of mucus in grams per meter of intestinal length, whereas the relative yield (%) can be calculated by dividing the mucus weight by total tissue weight before mucus collection for a specific length [157]. The pH measurements can be done on fresh mucus samples within 1 h of collection using a micro-electrode pH meter, while the water content can be analyzed by freeze-drying the mucus samples and comparing its weight with fresh samples for similar quantities [158]. Glycomic, lipidomic and metabolomic analyses can be performed like proteomic analysis by using mass spectrometry techniques like LC-MS or GC-MS [158]. Hence, it can be concluded that the mucus compositional changes occurring with respect to age and disease status of the host is a key research area during infection diseases and host-pathogen interaction studies.

Unveiling the immunological role of intestinal mucosa in viral gastroenteritis: pathways for future research

Intestinal mucosa serves as the frontline in between invading pathogens and the intestinal immune system. Intestinal mucosal immunity involves a complex interplay of cells like PCs, enterocytes, Goblet cells (GCs), innate lymphoid cells, intraepithelial lymphocytes, and lymphoid systems such as the ileal Peyer's patches, which together form the gut's innate immune system and regulate adaptive responses upon interaction with microbes [159]. Immune cells like dendritic cells (DCs) and neutrophils are in direct contact with the intestinal lumen, while specialized cells like γδ T receptor-expressing intraepithelial lymphocytes do not have access to the intestinal lumen but maintain homeostasis by producing AMPs and limiting pathogen invasion after barrier breaches [160]. CXCR1 + chemokine receptor 1 (CXC3CR1) expressing DCs can intercalate between epithelial cells to uptake antigens from luminal mucus, while chemosensory tuft cells, crucial for helminth infections, and M cells, which also uptake luminal antigens, may also be present in the intestinal mucus [161, 162]. Intestinal epithelial cells and local innate immunity form a barrier to pathogens via Toll-like receptors (TLRs), while adaptive immunity is established through antigen recognition by antigen-presenting cells (APCs) in Peyer's patches and mesenteric lymph nodes [163]. TLR1, TLR2, TLR4, TLR6, and TLR10 are specifically related to the recognition of viral proteins [164]. The T and B lymphocytes of the gut-associated lymphoid tissues (GALTs) are involved in IgA responses, further strengthening the adaptive immune response [165]. IgA is known to bind bacteria or viruses and slow down their diffusion and bacterial motility in the mucus [29]. Antimicrobial components such as lysozymes, defensins, and DMBT1 are also present in small intestinal mucus produced by PCs located in the intestinal crypts [30]. In humans, intestinal epithelial cells produce REG3A, which has bactericidal properties, typically against Gram-negative bacteria [31]. Additionally, GCs secrete immune modulators such as CLCA1, FCGBP, AGR2, ZG16, KLK1, and TFF3, though their specific functions remain largely unknown [9]. Hence, significant gaps remain in our understanding of how these immunological factors of the intestinal mucosa interact with enteric viruses, offering abundant opportunities for future research.

The implications of mucin glycans for protection against pathogens

Among the various constituents of the mucosal system, mucins stand out as the primary functional elements of intestinal mucus, as reported in various studies [166–169]. Within the realm of mucins, it is the mucin glycans that present the most compelling and relatively unexplored area of study in relation to enteric viral infections. TGEV has an affinity for different sialic acids, such as Neu5Gc, Neu5Ac, and Neu5,9Ac2, which also act as receptors or receptor binding co-factors for other coronaviruses [26, 27]. MUC2 is rich in these sialic acids and may play a role in determining virus pathogenicity [170, 171]. The sialic acids are known to bind and trap respiratory viruses aiding in their clearance via mucociliary transport of the respiratory system [172]. Other viruses like rotavirus, influenza B and C viruses, respirovirus and certain parvoviruses are also known to use sialic acids as receptors through their sialolectins [173], where mucin glycans might be of interest as they may provide decoy receptors for these viruses. The glycan-dependent inhibitory effect of mucins against coronavirus infection of live cells is also reported [138]. Thus, performing a glycan analysis and subsequently using them in virology assays can provide a good idea about their potential protective role against enteric viruses. Commercially available mucins like porcine gastric mucin are available to carry out these studies [174]. However, the ex vivo mucus extracted directly from the host species can also be purified to extract mucins. Addition of guanidine hydrochloride or urea in the extracted mucus can weaken the hydrophobic bonds between mucins and other mucosal components easing subsequent purification [175, 176]. Using density gradients like cesium chloride or rate zonal centrifugation, further impurities like cellular and fecal debris can be removed [177–179]. Chromatographic separation like size exclusion chromatography or gel filtration can be employed to isolate relatively larger sized mucins from other mucosal components [180]. Mucin fractions isolated under this fashion can be monitored by UV absorbance at 215 nm and visualized by mucin-specific staining like PAS [181]. These isolated mucins can then be desalted, concentrated, and lyophilized for storage, while mass spectrometry can be used to assess their composition [182]. From these purified mucins, *O*-glycans can be extracted using non-reductive alkaline β-elimination ammonolysis, which conserves the structure of glycans [183]. From these extracted glycans, core glycan structures can be isolated using partial acid hydrolysis and analyzed by mass spectrometry [184]. These glycans can be incorporated into virology experiments like virus plaque-blocking assays. Concludingly, mucin glycans isolated from different host species can give an idea of the glycomic changes that might play a key role in protecting against enteric viral infections.

Use of intestinal mucus as a therapeutic measure against enteric infections

Although the idea of using purified mucins extracted from intestinal mucus as a therapeutic tool to combat enteric infections, particularly in newborn humans

and animals, may seem far-fetched, there is promising research in this area. However, the use of synthetic mucins as therapeutics is a viable possibility. For instance, Nason et al. have developed special HEK293 cells that are 'glyco-engineered' to express representative short tandem repeats (~200 amino acids) of human *O*-glycodomains [185]. This approach could potentially be adapted to enhance the therapeutic application of mucins. With tunable structures and patterns of *O*-glycans, that are representative of actual mucin repertoire of the body, large quantities of specific mucin glycodomains can be sustainably manufactured from natural mucin polymers and their therapeutic potential as dietary pre-biotic material can be assessed. These mucins can either offer their protective ability by directly blocking the invading viruses or bacteria, or by stimulating the natural commensals of the body in maintaining gut health.

Recombinant *N*-glycoproteins such as IgG and erythropoietin have already been investigated and characterized for their use as biopharmaceutical products [186–188]. Wohlschlager et al.. also described multiple *N*-glycans and up to 26 *O*-glycans attached to the chimeric TNF-α receptor fusion protein with a therapeutic potential [187]. Thus, similar quality control studies can be utilized for mucin *O*-glycan standardization. However, an important consideration here is that the therapeutic effect of mucus can be specific to host species, and the pathogens adapted to that species. Bovine mucins inhibited the infection of bovine-originated human coronavirus OC43 in susceptible cells in a concentration- and glycan-dependent manner but could not inhibit Mouse Hepatitis Virus (MHV), a mouse coronavirus [138]. This suggests that mucin biophysics and biochemistry is dependent on small conformational and glycoform changes that may be related to species genetics, metabolism, or the environment [12, 189, 190]. Hence, isolation of species-specific mucins and mucin glycans should be considered. Regardless, it is ambitious but an achievable idea that offers huge research potential. Apart from mucins, other antimicrobial components of mucus like AMPs or lipids also hold potential for synthetical engineering and be used as therapeutics. Moreover, with the progress in fecal microbiota transplantation and its success in treating gastrointestinal disorders [191], there is a potential for intestinal mucus transplants from healthy individuals to those with viral gastroenteritis, which should be explored.

Decoding viral pathogenesis and tropism through comparative mucus studies across different systems

Mucus composition varies across different physiological systems with a species [192]. This is another potentially important area to study viral pathogenesis and tropism along with changes or shifts occurring in them. For example, in 1995, key deletions in TGEV at nucleotides 621–681 gave rise to porcine respiratory coronavirus which mainly causes respiratory symptoms [193]. The variations in mucus structure and mucosal components between the two systems may have driven TGEV to mutate, enabling it to shift its virus tropism to PRCV. Previously in our lab, we analyzed the interaction of pseudorabies virus (PRV, diameter = ~250 nm) with porcine respiratory mucus using SPT [194]. Only PEGylated particles displayed chaotic Brownian movement in respiratory mucus, while negatively and positively charged particles, and negatively charged PRV were restricted. However, our study in the interaction of TGEV and similar-sized control particles showed that both the carboxylated (-) and PEGylated (=) particles showed chaotic Brownian movement in the intestinal mucus of 3-day and 3-week-old pigs [109]. This is likely due to the different composition of respiratory and intestinal mucus. Respiratory mucus contains mainly the MUC1/MUC4/MUC5AB-C complex, while MUC2 is the primary gel-forming mucin of small intestinal mucus [195]. Furthermore, the diversity of the host's immune components and microflora in mucus from both systems is critical in combating the invading pathogens [196]. Another study from our lab showed that PRV showed more penetration through porcine respiratory mucus at 4 °C compared to 37 °C after 30 min [197]. However, our study on TGEV in the case of 3-week intestinal mucus showed that its movement in both short- and long-duration diffusion assays showed was hindered at lower temperatures [109]. This indicates that intestinal and respiratory mucus behave differently when temperature is decreased, further highlighting the compositional differences between the two. Thus, similar studies can be designed for PRCV in terms of respiratory mucus and cross examining of both viruses in both types of mucus systems. The interaction of PRCV with the respiratory mucus along with availability of a different repertoire of available mucin glycans can provide a new angle on the shift in tissue tropism. Combining the analysis of physiochemical properties of porcine respiratory mucus, particle-like behavior of PRCV in respiratory mucus and assessing its blocking activity offers a lot of opportunities for further research. In Fig. 3, a schematic outline of the key compositional differences between mucus from the respiratory and gastrointestinal systems is presented to understand and aid in designing cross-system mucus studies toward unravelling the TGEV/PRCV tissue tropism.

Conclusion

The exploration of intestinal mucus as a defense against viral gastroenteritis opens numerous promising research pathways. The implications of mucin glycans for pathogen protection and the potential therapeutic use of intestinal mucus highlight the multifaceted role of mucus in

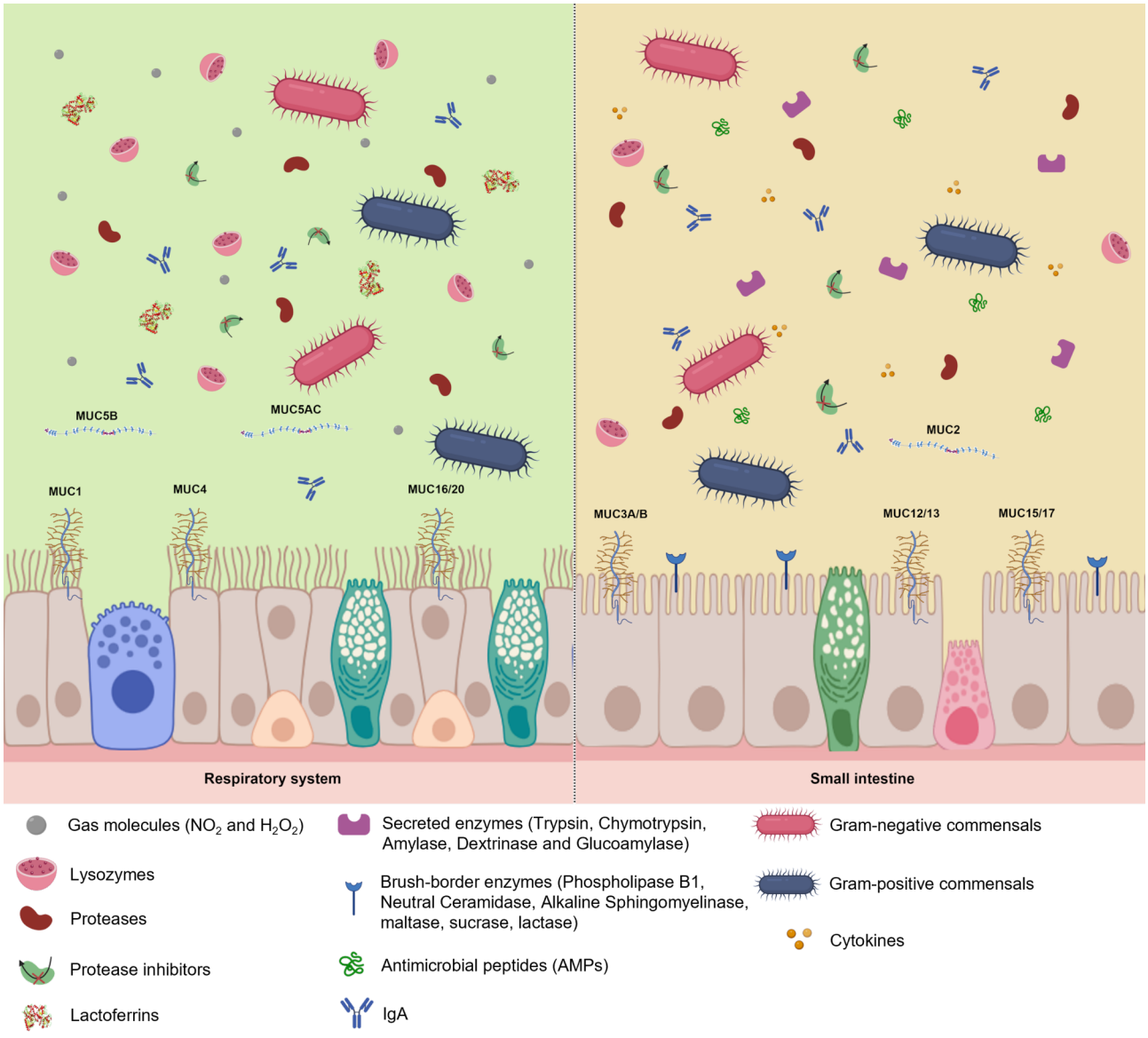

Fig. 3 Schematic overview of mucus compositional differences between the respiratory system and the small intestines. Small gas molecules and lactoferrins are predominant in the respiratory mucus while the cytokines and secreted digestive enzymes are more common in the small intestinal mucus. Adapted from Bansil et al. [30]

combating enteric infections. Comparative studies across different physiological systems further enhance our understanding of viral pathogenesis and tropism. Future research should focus on the detailed characterization of mucus components, including proteins, lipids, immunological factors and glycans, to uncover their specific roles in host-pathogen interactions. Additionally, the development of novel therapeutic approaches utilizing purified mucins or engineered glycodomains could revolutionize the treatment of enteric infections, particularly in vulnerable populations such as newborns and young animals. Overall, advancing our knowledge of intestinal mucus and its interactions with enteric viruses will not only deepen our understanding of viral gastroenteritis but also pave the way for innovative strategies to prevent and treat these infections. This dynamic field of research holds great potential for improving gut health and combating viral diseases.

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Author contributions
WS and HN conceptualized the work. WS, AA, and MT wrote the manuscript. WS and HN reviewed the manuscript. HN supervised the manuscript. All authors agreed to the final form of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The euthanization of pigs used in the case study (Sect. 4) was done in agreement with European legislation on animal experiments. All experimental procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine (LA1400076) of Ghent University, and all methods were carried out in accordance with the approved guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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