https://doi.org/10.1038/s44298-025-00164-3

Fucose dependent rotavirus and norovirus require fucosidase activity for optimal replication

Check for updates

Nazaret Peña-Gil[1,2], Nanci Santos-Ferreira[3], Sonia Llanos-Villatoro[1], Ana Melero[4], Roberto Cárcamo-Calvo[1,2], Sergi López-Navarro[1,2], Cristina Santiso-Bellón[1,2], Javier Buesa[1,2], Vicente Monedero[5], Maria J. Yebra[5], Joana Rocha-Pereira[3], Roberto Gozalbo-Rovira[1,2] & Jesús Rodríguez-Díaz[1,2] ✉

Rotavirus (RV) and norovirus (NoV) are enteric viruses responsible for acute gastroenteritis that require fucosylated histo-blood group antigens for infection in humans. How the interaction of these viruses with fucosylated glycans modulates infection is not well understood. Treatment of target cells with a bacterial α1,2 fucosidase enzyme reduced RV and NoV infection in vitro, but increased replication in vivo. Conversely, the fucosidase inhibitor 1-deoxyfuconojirimycin impaired viral replication in both models, highlighting the role of fucosidase activity in fucose-dependent enteric virus infection. This underscores the complexity of fucose interactions for these viruses and implicates fucosidase activity as a potential antiviral target for RV and NoV.

Despite a significant reduction in diarrhoeal-related deaths over the past two decades (from 2.6 million in 2000 to 1.5 million in 2019) diarrhoeal diseases remain a leading cause of death among children under five, particularly in low- and lower-middle-income countries[1]. Gastrointestinal viruses such as human rotavirus (RV) and human norovirus (NoV) are major contributors to acute gastroenteritis[1]. Therefore, a greater understanding of the biology of these viruses and the discovery of new therapeutic interventions are of key relevance.

Histo-blood group antigens (HBGAs) have been identified as key receptors or co-receptors involved in the infection processes of both RV and NoV[2]. These complex carbohydrates are expressed not only on the surface of red blood cells but also on mucosal surfaces of the respiratory, genitourinary, and gastrointestinal tracts[3]. HBGA biosynthesis involves the stepwise addition of monosaccharides to precursor structures by glycosyltransferases encoded by three main gene families: secretor (FUT2), Lewis (FUT3), and ABO. FUT2 encodes an α1,2-fucosyltransferase and certain alleles can lead to reduced or absent expression of specific HBGAs structures. Individuals lacking functional FUT2 are termed "non-secretors". FUT2 polymorphisms appear to have the most significant impact on susceptibility to natural RV and NoV infections in humans[4], indicating that the interaction of the viral lectin-like capsid domains with the HBGAs containing α1,2-fucoses are relevant for RV and NoV infections in humans.

Many respiratory viruses with hemagglutinin activity, such as influenza virus, also encode their own glycosidase enzyme; specifically, a viral neuraminidase. In influenza virus, these two activities (hemagglutinin and glycosidase) must be tightly balanced to ensure efficient influenza virus infection, as the enzymatic removal of sialic acid is essential for viral release and dissemination[5]. In contrast, although RV and NoV display hemagglutinin activity through their VP4 and VP1 proteins, respectively, they do not possess a virion-associated glycosidase, such as an α-L-fucosidase. Nevertheless, in the intestinal niche where RV and NoV replicate, fucosidase activity is present, either due to extracellular fucosidases secreted by various members of the gut microbiota as an adaptation to scavenge carbohydrates in this niche[6] or to the FUCA1 lysosomal fucosidase[7] expressed in the enterocytes.

Results

Impact of AfcA-mediated defucosylation on rotavirus and norovirus infection

Given the current gap in our understanding of how glycosidase activity might influence RV and NoV infections, we set out to investigate how the removal of α1,2-linked fucose from host glycans affects these viruses both in vitro and in vivo. For this purpose, we used the AfcA[6,8] enzyme, an extracellular α1,2 fucosidase from the intestinal bacterium *Bifidobacterium*

[1]Departamento de Microbiología, Facultad de Medicina, Universidad de Valencia, Valencia, Spain. [2]INCLIVA. Instituto de Investigación Sanitaría del Hospital Clínico de Valencia, Valencia, Spain. [3]KU Leuven, Department of Microbiology, Immunology and Transplantation, Rega Institute, Virus-Host Interactions & Therapeutic Approaches (VITA) Research Group, Leuven, Belgium. [4]Departamento de Farmacia y Tecnología Farmacéutica y Parasitología, Universidad de Valencia, Valencia, Spain. [5]Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Valencia, Spain. ✉e-mail: jesus.rodriguez@uv.es

bifidum. We first confirmed that AfcA effectively removed α1,2-linked fucose (a constituent of the H antigen) from monolayers of differentiated adult human intestinal enteroids (Supplementary Fig. 1). We next evaluated the effect of AfcA on viral replication in in vitro models. Infection by the cell culture-adapted human RV strain Wa (G1P[8]) in differentiated Caco-2 cells[9] (dCaco2) was significantly reduced in monolayers preincubated with AfcA (64.47 ± 15.91% reduction; $p = 0.0155$) (Fig. 1). Similarly, treatment of differentiated adult 3D human intestinal enteroids (3D HIEs)[10] (Supplementary Fig. 2) with AfcA, followed by infection with a stool filtrate positive for the human NoV GII.4_2012 Sydney strain (NoV_{43} isolate) resulted in a significant decrease in viral replication (37.31 ± 12.65% reduction; $p = 0.0419$) (Fig. 1). Using a highly sensitive cell viability assay confirmed that the in vitro reduction in viral replication was not due to cellular toxicity induced by the AfcA enzyme (Supplementary Fig. 3). Following these in vitro findings, we next assessed the impact of AfcA on RV and NoV infections in a mouse model[11,12]. Unexpectedly, fecal shedding of both RV_{Wa} and NoV_{43} (Fig. 1) was significantly higher in the animals pre-treated with AfcA across multiple sampling times, suggesting that fucosidase activity enhanced RV and NoV replication in vivo.

Inhibition of fucosidase activity impairs rotavirus and norovirus replication

To further validate these findings, we used the fucosidase inhibitor 1-deoxyfuconojirimycin (DFJ)[7], that was active against a panel of bacterial fucosidases from intestinal bacteria[6,13] (Supplementary Fig. 4) and did not show any in vitro toxicity (Supplementary Fig. 3). Compared to AfcA treatment, DFJ produced a stronger inhibition of the in vitro replication of both RV_{Wa} (95.30 ± 12.44% reduction; $p = 0.0016$) (Fig. 2) and NoV_{43} (78.67 ± 7.26% reduction; $p = 0.0004$) (Fig. 2). This inhibition was dose-dependent, with half-maximal effective concentrations (EC_{50}) of 67.72 ± 11.87 nM for RV_{Wa} and 140.14 ± 38.56 nM for NoV_{43} (Supplementary Fig. 5). Furthermore, in the mouse model, treatment with DFJ led to a significant reduction in RV_{Wa} fecal shedding by day 4 post-infection (Fig. 2). In the case of NoV, viral shedding ceased by day 3 in the DFJ-treated group, whereas it persisted until day 4 in the control group (Fig. 2). These results confirmed that inhibition of fucosidase activity impaired viral replication and shedding of both RV and NoV in vivo.

Discussion

Overall, our findings suggest that fucosidase activity may play an important role in the replication of both RV and NoV in vitro and in animal models. As neither of these viruses encode this enzymatic function, it must be supplied by the host or by the intestinal microbiota. Defucosylation of target HBGAs by AfcA in our in vitro experiments likely reduces the availability of RV and NoV receptors present in the cell surface (Fig. 3), thereby impairing infectivity in cellular models. Interestingly, this antiviral effect did not translate to the in vivo setting in a straightforward manner. In the more complex in vivo environment, increased fucosidase activity through AfcA administration had the opposite effects compared to in vitro conditions, leading to enhanced infectivity. In analogy to the coordinated action of hemagglutinin and neuraminidase during influenza virus infection[5,14], in vivo defucosylation may facilitate RV and NoV access to epithelial targets by reducing mucus trapping and facilitating viral infection (Fig. 3). Notably, experiments showing that DFJ diminishes infectivity both in vitro and in vivo suggest a combined contribution of both endogenous (host-associated) and exogenous (microbiota-associated) fucosidase activity in infection by fucose-dependent viruses.

A methodological constraint of our in vivo experimental model is the requirement to use antibiotic-treated mice to enable efficient replication of human RV[11] and NoV[12]. This approach likely results in a depleted intestinal microbiota, which in turn leads to reduced fucosidase activity within the gut. Nevertheless, the administration of AfcA fucosidase in this context, potentially compensating for the loss of microbiota-derived enzymatic function, led to enhanced viral infectivity. This finding suggests that the

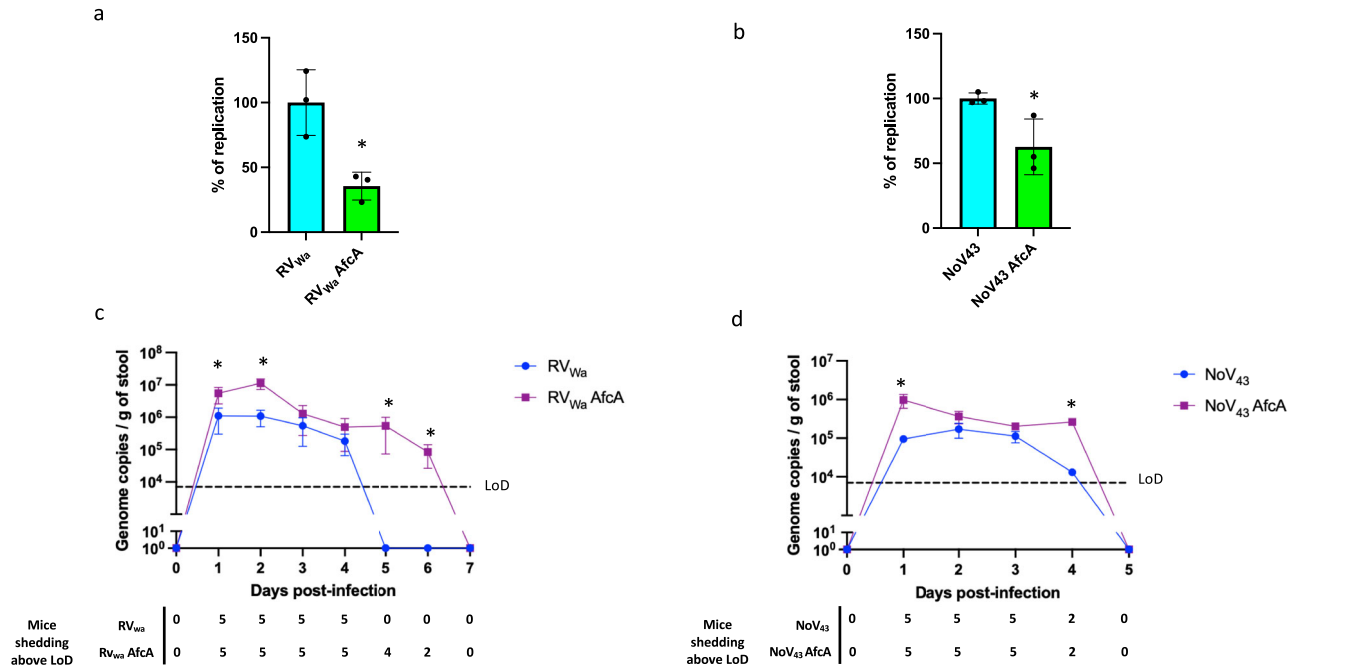

Fig. 1 | AfcA fucosidase reduces human RV and NoV replication in vitro but enhances viral shedding in vivo. a Percentage of replication relative to the control of human RV strain RV_{Wa} (G1P[8]) in differentiated Caco-2 cells and **b** NoV GII.4 Sydney 2012 (NoV_{43}) in 3D human intestinal enteroids (3D HIEs), pretreated or not with the α1,2 fucosidase AfcA (20 µg/well). Viral replication was quantified by RT-qPCR 48 h post-infection. Data represent means ± SEM from three independent experiments. **c** Fecal shedding of RV_{Wa} and **d** NoV_{43} in mice pretreated with AfcA (50 µg/mouse of liposome-formulated AfcA intragastrically administered daily for 2 days prior infection), compared to controls. Viral RNA was quantified by RT-qPCR from stool samples collected daily post-infection. Data show mean viral genome copies per gram of feces ± SEM of the mice shedding virus above the limit of detection (LoD) at each time point. The LoD is indicated by the dashed line. The number of mice shedding viruses above the LoD is indicated at each time point. Statistical analysis was performed using two-tailed unpaired *t*-test; $p < 0.05$ was considered significant and indicated with an asterisk (*).

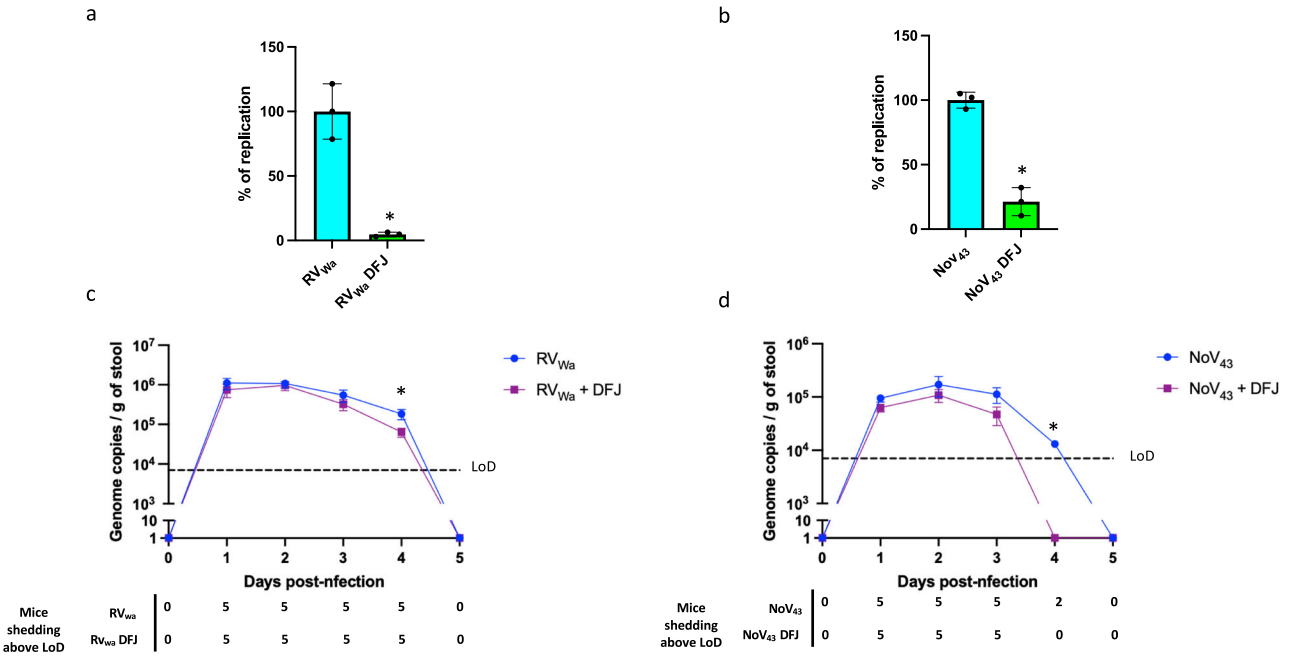

Fig. 2 | Inhibition of fucosidase activity by DFJ impairs RV and NoV replication in vitro and in vivo. Inhibition of RV_{Wa} **a** and NoV_{43} **b** replication in vitro by the fucosidase inhibitor 1-deoxyfuconojirimycin (DFJ) at a concentration of 800 nM. Viral replication was assessed by RT-qPCR at 48 h post-infection and is shown as a percentage relative to the untreated control. Fecal viral shedding of RV_{Wa} **c** and NoV_{43} **d** in mice treated with DFJ (100 µL of viral inoculum containing 20 mM DFJ per mouse) compared to untreated controls. DFJ administration significantly reduced RV shedding at day 4 post-infection, and shortened NoV shedding to 3 days. Data show mean viral genome copies per gram of feces ± SEM of the mice shedding virus above the limit of detection (LoD) at each time point. The LoD is indicated by the dashed line. The number of mice shedding viruses above the LoD is indicated at each time point. Statistical analysis was performed using two-tailed unpaired *t*-test; $p < 0.05$ was considered significant and indicated with an asterisk (*).

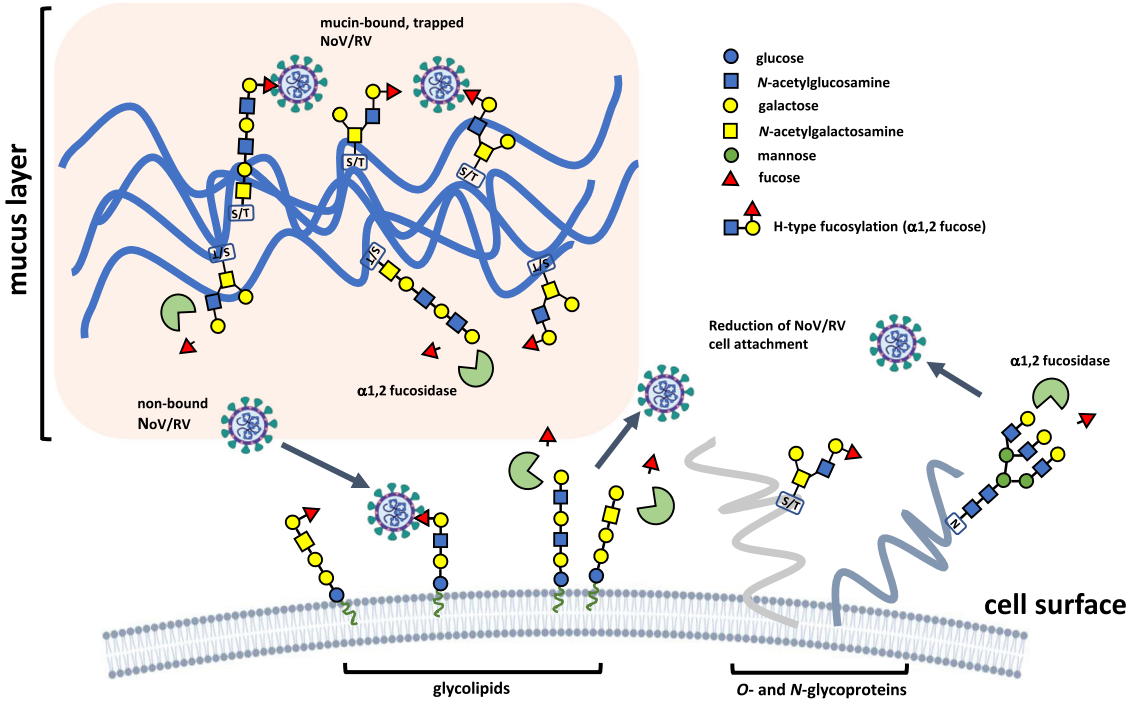

Fig. 3 | Hypothesis for the opposite effects of external α1,2-fucosidase addition on human RV and NoV replication in cell culture infection models versus mouse model. Human NoV and RV interact with H-type (α1,2-fucosylated; synthesized by FUT2 activity) HBGAs present in glycoproteins and glycolipids. In cell culture systems, fucosylated HBGAs on the cell surface facilitate viral attachment. In contrast, in the intestinal epithelium, the presence of highly fucosylated mucins in the mucus layer may trap virions, hindering access to target cells. Thus, removal of α1,2-linked fucose residues reduces infection in vitro but increases it in vivo.

added fucosidase can functionally substitute for microbiota-associated contributions. A key limitation of our findings is that the use of the DFJ inhibitor does not allow us to distinguish between the contribution of endogenous and exogenous fucosidase activity on RV and NoV infectivity. However, the observation that DFJ exerts inhibitory effects in vitro, where exogenous fucosidases are absent, strongly supports a role for the host fucosidase FUCA1 in promoting viral infectivity. Both RV and NoV utilize the endosomal pathway during infection, and FUCA1, which localizes to enterocyte lysosomes and to which DFJ was initially developed[7], may be functionally relevant in this context.

While we assessed the impact of AfcA treatment on defucosylation in HIEs that mimic the intestinal lumen architecture, the extent and spatial distribution of defucosylation across different regions of the mouse intestine following AfcA administration remain to be characterized. Moreover, beyond measuring increased viral shedding in feces, evaluating viral replication in distinct intestinal segments and correlating it with local fucosidase activity will improve our vision of the role of fucosidases in RV and NoV replication.

Our results shed some light on the fundamental mechanisms governing the interplay between the host, enteric viruses and the microbiota (a source of fucosidases). They highlight a novel contribution of an enzymatic activity that is critical for RV and NoV infection and which is likely to shape the host-pathogen-commensal interface. Targeting fucosidase activity to control enteric viral infection may offer promising new antiviral therapeutic strategies.

Methods

Expression and purification of glycosidases

Expression of the glycosidases Fuc2358[6], Fuc5372[6], AlfB[13], AlfC[13], AfcA[8], and LnbB[15] was carried out in *E. coli* BL21. Bacterial cultures carrying expression plasmids were grown overnight at 37 °C with agitation (250 rpm) in 5 mL of 2 × YT medium supplemented with 100 µg/mL ampicillin. The following day, the pre-culture was transferred into 1 L of fresh 2 × YT medium containing 100 µg/mL ampicillin and incubated under the same conditions until the OD_{600} reached approximately 0.5 (~ 4 h). Protein expression was induced by the addition of 0.1 mM IPTG, and cultures were incubated overnight at 18 °C with shaking. Cells were harvested by centrifugation at 11,100 × *g* for 30 min and resuspended in 15 mL of 1× PBS containing 0.015 g lysozyme, 3U DNase I, and 0.8 mM PMSF. After 30 min of incubation with agitation at room temperature, cell lysis was performed using 10 cycles of sonication (30 s on/30 s off). Cell debris was removed by centrifugation at 40,000 × *g* for 40 min. The clarified lysate was subjected to affinity chromatography using 5 mL HisTrap columns (Cytiva) on an ÄKTA Prime FPLC system (Cytiva). After initial equilibration with buffer A (1 × PBS, pH 7), the column was washed with buffer A containing 5% and 10% buffer B (1 × PBS containing 500 mM imidazole, pH 7), respectively, and proteins were eluted with 100% buffer B. Eluted fractions were analyzed by 10% SDS-PAGE. Buffer exchange was performed using 30 kDa Amicon centrifugal filters (Millipore), and purified proteins were resuspended in 1 × PBS containing 15% glycerol before storage at −80°C. Protein concentrations were determined using the Bradford assay.

Fucosidase inhibitor characterization

To determine the half-maximal effective concentration (EC_{50}) of the fucosidase inhibitor 1-deoxyfuconojirimycin hydrochloride (DFJ; Biosynth), 10 µg of each fucosidase were incubated with increasing concentrations of DFJ (0–1000 nM) for 1 h at 37 °C in 100 µL of 1 × PBS. Following incubation, 1 mM of the chromogenic substrate 4-nitrophenyl-α-L-fucopyranoside (pNP-Fuc) was added. After 15 minutes, the release of *p*-nitrophenol was quantified by measuring absorbance at 405 nm using a MultiSkan FC spectrophotometer. Absorbance values were used to calculate EC_{50} values via nonlinear regression analysis using the drc package (version 3.0.1) in R (version 4.1.2) under macOS Big Sur 11.7.6. Graphical representations were generated using ggplot2 (version 3.4.3). To evaluate the specificity of DFJ, 10 µg of the lacto-*N*-biosidase LnbB was incubated with varying concentrations of DFJ under the same conditions. Subsequently, 5 mM of the substrate 4-nitrophenyl-β-D-galactopyranoside (pNP-Gal) was added. After 15 minutes, absorbance at 405 nm was recorded to assess enzymatic activity.

Cell culture and maintenance

The cell lines Caco-2 and MA104 were cultured in minimum essential medium (MEM) supplemented with 1% penicillin and streptomycin (P/S), 1% glutamine, 1% non-essential amino acids, and 10% foetal bovine serum (FBS). Both cell types were maintained in T75 flasks at 37 °C in a humidified atmosphere with 5% CO_2. The media and supplements used were purchased from Gibco. When the cultures reached 80–90% confluency, they were washed with 7 mL of DPBS (Corning) and detached using 1 mL of verseno trypsin (Lonza) for 10 minutes at 37 °C. The cells were then resuspended with 9 mL of complete medium, and 2 mL of the suspension were transferred back into each flask. A 10 µL sample was taken for cell counting and viability assessment, which was performed by diluting the cells 1:1 with trypan blue solution (Sigma) and using dual chamber counting slides and an automated cell counter (TC20, Biorad). Subsequently, cells were seeded into flat bottom 96-well plates at 10^5 cells per well in a volume of 200 µL to ensure full confluency within 24 h. For Caco-2 cells, differentiation occurred 11 days after confluency, and experiments were conducted 14 days after confluence. During those 14 days, the media was changed three times. If differentiation was not required, cells were infected on the following day after confluency. Cells were used until passage 30 to ensure genetic stability.

Production of RV stock

African green monkey kidney epithelial cells (MA104 cell line) were used to propagate the RV Wa strain (RV_{Wa}), which belongs to the globally prevalent human genotype G1P[8]. MA104 cells were infected with RV_{Wa} at a multiplicity of infection (MOI) of 0.1 and processed, as previously described[16].

Infection dCaco-2 cells with RV_{Wa}

RV infection was performed in 96-well plates of differentiated cells, as previously described[9]. For each infection experiment, at least two plates were used. One plate served as the 1-hour time point to assess viral adhesion, while the other plates were used to determine viral replication. RV_{Wa} was thawed at room temperature and diluted at 10^5 pfu/mL in complete MEM medium without FBS. The dilution was supplemented with 10 µg/mL of trypsin IX (Sigma) to activate the virus. The viral dilution was prepared in either 96-well plates with round bottoms (for volumes below 250 µL) or sterile crystal tubes (for volumes above 250 µL) and incubated at 37 °C for 1 h. Meanwhile, the cells were washed twice with serum-free medium (MEM without FBS). Subsequently, 180 µL of serum-free medium, supplemented with 1 µg/mL of trypsin IX, were added to each well. After the viral activation period, 20 µL of the viral dilution were added to the corresponding wells. The cells were infected for 1 h at 37 °C with 5% CO_2. Following this incubation, the inoculum was aspirated and 100 µL of serum-free medium, supplemented with 1 µg/mL of trypsin IX, was added to each well. The plate designated as the 1-hour time point was frozen at -80 °C, while the other plates were further incubated at 37 °C with 5% CO_2 until the desired time point was reached. Each experiment was performed in triplicate.

Cellular treatment with AfcA fucosidase

In order to study the impact of fucosidases on RV replication, dCaco-2 cells were treated prior to infection with the AfcA enzyme. For this purpose, cells were washed twice with 200 µL of serum-free medium. Then, 20 µg of AfcA were added to each well in serum-free medium in a total volume of 200 µL. The cells were incubated for 2 h at 37 °C and 5% CO_2 and they were washed twice with complete medium without FBS, prior proceeding with infection as indicated above.

Norovirus detection, genotyping and filtration

Between 2019 and 2024, stool specimens were obtained from children with acute gastroenteritis attended at the Hospital Clínico Universitario de Valencia, Spain. One sample, designated NoV_{43}, tested positive for the GII.4 Sydney 2012 norovirus variant via RT-PCR[12]. Half g of this sample was transferred to a 15 mL conical tube and diluted in 4.5 mL 1 × PBS. The stool suspension was homogenized using a 1 mL pipette tip, followed by vortexing for 30 s. After a 5-min incubation at room temperature, the sample was vortexed again for an additional 30 seconds. Homogenates were then subjected to three cycles of sonication (30 seconds each, 1-minute intervals) using a Branson Sonifier 250 set to output level 7. Following sonication, the sample was centrifuged at 4,500 × *g* for 10 min at 4 °C to remove particulate debris. The resulting supernatant was transferred to a new 15 mL conical tube and centrifuged again under the same conditions to ensure clarity. The final supernatant was sequentially filtered through syringe filters with decreasing pore sizes: 5.0, 1.2, 0.8, 0.45, and 0.2 µm (Merck and Whatman). The resulting 10% (w/v) stool filtrate was aliquoted and stored at −80 °C until further use.

Human Intestinal Enteroids maintenance and differentiation

Human intestinal enteroids (HIEs) were generated *in house*[10]. HIEs were maintained as undifferentiated 3D HIEs domes embedded in Matrigel in organoid growth media (OGM, IntestiCult Organoid Growth Medium [STEMCELL] + 100 U/mL P/S + 5 µg/mL plasmocin). For infections, HIEs were differentiated by adding organoid differentiation media (ODM, Advanced DMEM/F12 + 2 mM GlutaMAX-1 + 10 mM HEPES + 100 U/mL P/S + 100 µg/mL Primocin + 50 ng/mL mouse recombinant EGF + 100 ng/mL mouse recombinant Noggin + 10 nM Lue15-Gastrin I + 500 nM A-83-01 + 1 mM N-acetylcysteine +1X B27 supplement + 1X N2 supplement + 5 µM DAPT) to the wells 4 days after splitting. Infection experiments were performed five days post-differentiation.

Immunofluorescence of 2D monolayers of HIEs treated with AfcA

Adult HIEs were seeded into 96 well-plates at a density of 10^5 cells/well. Once confluency was achieved after 3-5 days, ODM was added. After 4 days, each well was treated with 20 µg of AfcA. The fucosidase was resuspended in ODM in a total volume of 200 µL/well and incubation took place for 2 h at 37 °C and 5% CO_2. Afterwards, wells were washed twice with 200 µL of complete medium without growing factors (CMGF(-), Advanced DMEM/F12 + 2 mM GlutaMAX-1 + 10 mM HEPES + 100 U/mL P/S + 100 µg/mL Primocin) and fixated with 100 µL of ice cold MeOH at -20 °C for 20 min. Subsequently, cells were washed twice with 100 µL of PBS 1X and blocked with 100 µL of blocking buffer containing 3% BSA, 0.2% Triton X-100 and 1% goat serum for 30 min at room temperature. Once blocking was finished, cells were incubated with UEA-I lectin conjugated to fluorescein (Vector laboratories) at 1:1000 and DAPI at 1:1000 dilution for 1 h at room temperature in agitation and protected from light. Finally, each well was washed twice with 100 µL of PBS and maintained in PBS. Cells were observed using Leica DMi8 inverted fluorescence microscope (Leica Microsystems).

Viability assays

The AfcA fucosidase and fucosidase inhibitor DFJ toxicity was tested in dCaco-2 cells and HIEs. Once 3D HIEs were differentiated, collected, counted, and distributed in 15 mL tubes, AfcA (10 and 20 µg/well) or DFJ (concentrations ranging from 0 to 15 µM) were added. After incubation for 2 h at 37 °C and 5% CO_2, cells were seeded in a 24-well plate, incubated for 48 h, and cell viability was assessed using the CellTiter-Glo® 3D Cell Viability Assay Kit (Promega), following manufacturer's instructions. For dCaco-2 cells the same concentrations of AfcA and DFJ were added to cells differentiated for 11 days, following incubation for 2 h at 37 °C and 5% CO_2. Viability was assayed with the same kit 48-h post treatment.

Infection of 3D HIEs with NoV_{43}

The infection of differentiated 3D HIEs with NoV was performed, as previously described[10]. Briefly, at the infection day, 3D HIEs domes were collected in a 15 mL falcon tube treated with AARS (anti-adherence rinsing solution) and washed 3 times with CMGF(-) to remove Matrigel. Afterwards, HIEs were pelleted and resuspended in 2 mL of NoV infection medium. A 100 µL aliquot was pelleted, resuspended in TryplE, incubated for 20 min at 37 °C to obtain single cells, and counted. A total 4–10^5 cells were used. The desired amount of HIEs were distributed in 15 mL tubes treated with AARS or low binding microtubes up to 2 or 1 mL of incubation media, respectively. The NoV filtrate was added to the cells at a MOI of 1 and incubated for 2 h at 37 °C and 5% CO_2, cell suspension was resuspended every 30 min with wide bore tips. Then, cells were washed 3 times with CMGF(-), pelleted and platted in Matrigel domes.

NoV Infection of 3D HIEs treated with AfcA

It was also studied the effect of the AfcA fucosidase in NoV infections of 3D HIEs before infection. After cell count and distribution in 15 mL tubes, 20 µg of AfcA was added. After 2 h incubation at 37 °C and 5% CO_2 with occasional mixing, cells were washed 3 times with CMGF (-), resuspended in the correspondent infection media and proceed with the infection as described above.

Effect of fucosidase inhibitor on in vitro viral infection

To investigate the effect of a fucosidase inhibitor in viral replication, DFJ was employed. It was diluted in MQ water. During RV activation, the inhibitor was added to the viral dilution at the desired concentrations and incubated for 1 h at 37 °C. For NoV infections the viral stock was incubated in ODM for 1 h at 37 °C previous to viral infection. Then, viral infections were performed as described above.

RNA extraction, RT-qPCR and $2^{-\Delta\Delta Ct}$

The plates used in RV infections were thawed at 37 °C, and subsequently the supernatant and cells were collected in a microcentrifuge tube. RNA was extracted from the collected samples following the instructions of the Viral RNA Extraction Kit (Macherey Nagel). To extract RNA from NoV infections in HIEs, tubes containing infected cells and lysis buffer were thawed at room temperature. RNA was extracted with the RNAeasy mini kit (Qiagen). To determine the viral replication, qPCR analysis was conducted using an Exycler 96 instrument (Bioneer) or QuantStudio 5 (AppliedBiosystems). The qPCR master mix consisted of NZYSpeedy qPCR Probe Master Mix at a concentration of 1 × (NZY), forward and reverse primers and a probe specific to RV[11] or NoV[12], respectively at a concentration of 0.25 µM, RT SSIII enzyme (20 U/sample, Invitrogen), and DEPC-treated water (Invitrogen). Specific forward and reverse primers and a hypoxanthine phosphoribosyltransferase 1 (HPRT1) probe at a concentration of 1 × (IDT) were used for normalization. The following cycling conditions were applied: 30 min at 50 °C, 10 s at 95 °C and 45 cycles of 15 s at 95 °C and 1 minute at 60 °C. The replication of the virus was quantified using the $2^{-\Delta\Delta Ct}$ method. To facilitate interpretation, all results are expressed as the percentage of replication relative to the control in each experiment.

Preparation of liposomes containing AfcA for in vivo experiments

The fucosidase AfcA was administered to mice in liposomes to ensure its protection upon passage through the stomach. The enzyme-containing lipid vesicles were fabricated with the solvent casting method. A mixture of 0.45 g of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Lipoid) and 12 mg of cholesterol (Acofarma) as surfactant were solved in 50 mL of methanol. The solution was then distilled using a rotatory evaporator (BUCHI) for 12 h at 55 °C and 250 bar. The resulting lipidic layer was reconstituted with 5 mg of lyophilized AfcA dissolved in 10 mL of PBS for 1 h at 30 °C. Control liposomes were prepared following the same protocol in the absence of AfcA.

Infection experiments in mice

Female C57BL/6 mice (6–8 weeks old) were acclimated for one week in individually ventilated cages (five per cage) under controlled temperature (22 ± 2 °C), humidity (55 ± 10%), and a 12 h light/dark cycle, with ad

libitum access to food and water. Mice were randomly allocated into three experimental groups (Control, AfcA-treated, DFJ-treated). Investigators were not blinded during experimental procedures or data analysis. No animals or samples were excluded from analysis. To allow infection by human RV_{Wa} and NoV_{43} viruses, the mice gut microbiota was depleted with an antibiotic cocktail (1 g/L ampicillin, 1 g/L metronidazole, 1 g/L neomycin, and 0.5 g/L vancomycin) administered ad libitum in the drinking water, as previously described[17]. This cocktail was refreshed every three days throughout the entire experiment. Mice were fed a purified, defined germ-free diet (AIN-93G; Envigo, Barcelona, Spain). Three weeks after initiating the antibiotic treatment, infection experiments were performed as previously described[11,12]. Six groups of mice (three groups for each virus) were infected orally with either RV_{Wa} (1×10^8 genome equivalents in 120 µL PBS) or NoV_{43} (1×10^8 genome equivalents in 120 µL PBS). The experimental groups were as follows:

Control group: Mice received antibiotics for three weeks and were then infected with RV_{Wa} or NoV_{43}. Mice received control liposomes without AfcA (150 µL total volume) on days -2 and -1 prior to infection.

AfcA group: Mice were treated as in the control group, but in addition received 50 µg of AfcA enzyme encapsulated in liposomes (150 µL total volume) on days -2 and -1 prior to infection.

DFJ group: Mice were treated as in the control group, but the viral stocks were prepared in PBS supplemented with 20 mM DFJ and the mix of inhibitor and virus given simultaneously.

Both the viruses and the AfcA enzyme were administered using a flexible feeding tube. Fecal samples were collected daily from day 0 (prior to infection) until day 7 post-infection, and stored at −80 °C until viral RNA extraction.

Quantification of RV and NoV from stool samples by RT-qPCR

RNA was extracted from fecal samples with the Viral RNA extraction kit (Macherey Nagel). First, feces were weighted in order to finally normalized the results depending on feces quantity. After that, 700 µL of the lysis solution was added to the 80 mg of stool samples, which were then sonicated for 45 s using a Branson Sonifier 250, with output set at 2. Later, samples were incubated at 70 °C for 5 min and centrifugated at 21,000 g for 5 min. RNA from the supernatant was then extracted following manufacturer's instructions. RNA was diluted 1/10 and then the RT-qPCR was performed using the StepOne 48 wells thermocycler (AppliedBiosystems), with primers and probes corresponding to RV[11] and NoV[12]. The standard curve was generated by serial end-point dilution, amplifying 10-fold dilutions of the quantified plasmid containing the RV or NoV target sequence by RT-qPCR in triplicates. The results are presented as genome copies per gram of stool.

Statistical analysis

Graphs are represented as means ± SEM. Statistical differences were determined with the GraphPad Prism 8.0.1 software, using the unpaired t-test to compare the means between two distinct groups. A p value ≤ 0.05 was considered significant.

Ethics statement

All mouse-related experiments were carried out at the University of Valencia's Animal Production and Experimentation Service in accordance with both national and international guidelines. The protocols received approval from the Ethics Committee for Animal Welfare as well as the "Dirección General de Agricultura, Ganadería y Pesca" of the "Generalitat Valenciana" (approval file: 2018/VSC/PEA/0181, dated 19 December 2018). Animals were anesthetized with halothane and euthanized by cervical dislocation at the end of the experiments, following institutional and European ethical guidelines. Reporting of animal experiments follows the ARRIVE Essential 10 guidelines[18]. Detailed information on study design, randomization, blinding, animal housing, and procedures is provided in Supplementary Table 1.

Ethical approval for the use of human stool samples was granted by the University of Valencia's Human Research Ethics Committee (registration number H154401046838).

Data availability

The datasets generated and analyzed during the current study have been deposited in the institutional repository of the Universitat de València (RODERIC) and are publicly available at https://hdl.handle.net/10550/116966.

Code availability

No custom code was used in this study.

Received: 8 September 2025; Accepted: 18 November 2025;
Published online: 29 November 2025

References

1. World Health Organization. The top 10 causes of death. (2024). Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death. (Accessed: 19th2024)
2. Peña-Gil, N. et al. The role of host glycobiology and gut microbiota in rotavirus and norovirus infection, an update. *Int. J. Mol. Sci.* **22**, 13473 (2021).
3. Marionneau, S. et al. ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world. *Biochimie* **83**, 565–573 (2001).
4. Rodríguez-Díaz, J. et al. Relevance of secretor status genotype and microbiota composition in susceptibility to rotavirus and norovirus infections in humans. *Sci. Rep.* **71**, 1–10 (2017).
5. Wallace, L. E., de Vries, E., van Kuppeveld, F. J. M. & de Haan, C. A. M. Neuraminidase-dependent entry of influenza A virus is determined by hemagglutinin receptor-binding specificity. *J. Virol.* **97**, e00602–e00623 (2023).
6. Moya-Gonzálvez, E. M. et al. Infant gut microbial metagenome mining of α-l-fucosidases with activity on fucosylated human milk oligosaccharides and glycoconjugates. *Microbiol. Spectr.* **10**, e0177522 (2022).
7. Kato, A. et al. Synthesis and biological evaluation of N-(2-fluorophenyl)-2β-deoxyfuconojirimycin acetamide as a potent inhibitor for α-l-fucosidases. *Bioorg. Med. Chem.* **21**, 6565–6573 (2013).
8. Ashida, H. et al. Two distinct α-L-fucosidases from Bifidobacterium bifidum are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. *Glycobiology* **19**, 1010–1017 (2009).
9. Peña-Gil, N. et al. Culture of human rotaviruses in relevant models shows differences in culture-adapted and nonculture-adapted strains. *Int. J. Mol. Sci.* **24**, 17362 (2023).
10. Mirabelli, C. et al. Human norovirus efficiently replicates in differentiated 3D-human intestinal enteroids. *J. Virol.* **96**, 1–12 (2022).
11. Gozalbo-Rovira, R. et al. Microbiota depletion promotes human rotavirus replication in an adult mouse model. *Biomedicines* **9**, 1–14 (2021).
12. Santiso-Bellón, C. et al. Replication of human norovirus in mice after antibiotic-mediated intestinal bacteria depletion. *Int. J. Mol. Sci.* **23**, 10643 (2022).
13. Rodriguez-Diaz, J., Monedero, V. & Yebra, M. J. Utilization of natural fucosylated oligosaccharides by three novel alpha-L-fucosidases from a probiotic Lactobacillus casei strain. *Appl. Environ. Microbiol.* **77**, 703–705 (2011).
14. Liu, M., de Vries, E. & de Haan, C. A. M. Virion motility of sialoglycan-cleaving respiratory viruses. *Npj viruses* **3**, (2025).
15. Gotoh, A. et al. Novel substrate specificities of two lacto-N-biosidases towards β-linked galacto-N-biose-containing oligosaccharides of globo H, Gb5, and GA1. *Carbohydr. Res.* **408**, 18–24 (2015).

16. Arnold, M., Patton, J. T. & Mcdonald, S. M. *Culturing Rotaviruses* **1**, 29 (2012).
17. Rubio-Del-Campo, A. et al. Infant gut microbiota modulation by human milk disaccharides in humanized microbiome mice. *Gut Microbes* **13**, 1–20 (2021).
18. du Sert, N. P. et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLOS Biol.* **18**, e3000410 (2020).

Acknowledgements

We thank Dr. Takane Katayama for providing the *E. coli* strains expressing the AfcA and LnbB enzymes. We also thank Dr. Ron Geller for critical reading of the manuscript and fruitful discussions.

Author contributions

J.R.-D., J.B., J.R.-P., V.M., and M.J.Y. conceived and designed the study. J.R.-D. and V.M. wrote the initial draft of the manuscript. J.R.-D., R.G.-R., J.R.-P., and M.J.Y. were responsible for funding acquisition. A.M. prepared the AfcA liposomes. N.P.-G., S.L.-V., S.L.-N., and R.C.-C. produced, purified, and characterized the recombinant glycosidases. N.P.-G., C.S.-B., and S.L.-V. conducted the experiments in dCaco-2 cells and performed viral quantification by qPCR. N.S.-F. and N.P.-G. carried out the experiments with human intestinal enteroids. R.G.-R. and N.P.-G. performed the experiments in mice. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s44298-025-00164-3.

Correspondence and requests for materials should be addressed to Jesús Rodríguez-Díaz.

Reprints and permissions information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

© The Author(s) 2025