

## PAPER

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# Human milk oligosaccharides and non-digestible carbohydrates prevent adhesion of specific pathogens *via* modulating glycosylation or inflammatory genes in intestinal epithelial cells

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Human milk oligosaccharides (hMOs) and non-digestible carbohydrates (NDCs) are known to inhibit the adhesion of pathogens to the gut epithelium, but the mechanisms involved are not well understood. Here, the effects of 2'-FL, 3-FL, DP3–DP10, DP10–DP60 and DP30–DP60 inulins and DM7, DM55 and DM69 pectins were studied on pathogen adhesion to Caco-2 cells. As the growth phase influences virulence, *E. coli* ET8, *E. coli* LMG5862, *E. coli* O119, *E. coli* WA321, and *S. enterica* subsp. *enterica* LMG07233 from both log and stationary phases were tested. Specificity for enteric pathogens was tested by including the lung pathogen *K. pneumoniae* LMG20218. Expression of the cell membrane glycosylation genes of galectin and glycocalyx and inflammatory genes was studied in the presence and absence of 2'-FL or NDCs. Inhibition of pathogen adhesion was observed for 2'-FL, inulins, and pectins. Pre-incubation with 2'-FL downregulated *ICAM1*, and pectins modified the glycosylation genes. In contrast, *K. pneumoniae* LMG20218 downregulated the inflammatory genes, but these were restored by pre-incubation with pectins, which reduced the adhesion of *K. pneumoniae* LMG20218. In addition, DM69 pectin significantly upregulated the inflammatory genes. 2'-FL and pectins but not inulins inhibited pathogen adhesion to the gut epithelial Caco-2 cells through changing the cell membrane glycosylation and inflammatory genes, but the effects were molecule-, pathogen-, and growth phase-dependent.

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## 1. Introduction

Breastfeeding is the gold standard for nutrition and healthy development of infants.<sup>1</sup> The advantages of breastfeeding have been widely recognized, which include lower risk for infections and reduction of inflammatory events in infants.<sup>2</sup> Human milk oligosaccharides (hMOs) are considered to be responsible for these beneficial effects. However, in about 70% of infants, breastfeeding is not an option for the whole period of lactation.<sup>3</sup> In those cases, cow milk-derived infant formula is given.<sup>4</sup> Cow milk lacks hMOs and therefore lacks some unique features of human milk. To compensate for some of the missing hMO functions in cow milk derived infant formula, non-digestible

carbohydrates (NDCs) such as inulins, galacto-oligosaccharides and pectins are applied.<sup>5,6</sup> In recent years, some synthetic hMOs have been applied in infant formula as a new emerging technique allowing for cost-effective production.<sup>7</sup>

An important function of hMOs and NDCs in infant formula is the reduction of pathogen infection.<sup>7</sup> A possible mechanism by which this is accomplished is through direct modulation of the intestinal epithelial glycosylation receptors.<sup>8,9</sup> The glycocalyx layer covering the intestinal epithelium is known to be an anchoring point for pathogens and has been reported to change in response to molecules such as hMOs.<sup>10</sup> The intestinal epithelial glycocalyx is built up of a core protein of glypicans linked to the cell membrane and further branched with glycosaminoglycan side chains of hyaluronic acid (HA) or heparan sulfate (HS).<sup>11</sup> HA and HS play an important role in the binding of pathogens and other antigens in the gut lumen.<sup>12,13</sup> Within the glycocalyx layer, galectins form a large family of lectins with affinity for *N*-acetylglucosamine residues and regulate the inflammatory responses by mediating protein–glycan and protein–protein interactions.<sup>14</sup> Among the 15 identified galectins in the glycocalyx, galectin-1 is a 'proto-type' galectin with one carbohydrate recognition domain (CRD) and it facilitates the secretion of anti-inflammatory cytokines.<sup>14</sup>

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Galectin-9 is a 'tandem repeat-type' galectin with two CRDs,<sup>15</sup> and was reported to be a target for the treatment of intestinal inflammatory diseases.<sup>16</sup>

During the pathogenesis of infection of gut epithelial cells, many other molecules are also involved in the inflammatory responses.<sup>17,18</sup> Interleukin adhesion molecule-1 (ICAM-1) is such a molecule. It is a trans-membrane glycoprotein in epithelial cells, which is reported to be highly expressed during inflammation<sup>18</sup> and to recruit leukocytes.<sup>19</sup> The chemokine CXC motif ligands and human beta defensin have also been reported to play an active role in the development of intestinal epithelial inflammation.<sup>17,20</sup> For example, higher incidence of necrotizing enterocolitis is associated with increased *CXCL1* expression in mice.<sup>21</sup> Infection and inflammation usually occur after interaction between the gut epithelium and specific, but to be identified, bacterial virulence and adhesion molecules on pathogens.<sup>22,23</sup> It is well known that expression of these adhesion receptors depends on the growth phase of the pathogen.<sup>24</sup> Pathogens from the stationary phase have a thicker peptidoglycan layer, which contribute to biofilm formation and stronger resistance to environmental stresses compared to pathogens from the log phase.<sup>24,25</sup> As in the intestine, pathogens may be in both phases, depending on the stage of the infection,<sup>26</sup> thus it is important to study the anti-adhesion effects of hMOs and NDCs after treating the gut epithelium with pathogens from both the log and stationary phases.

As there is limited information available about how hMOs and NDCs modulate gut epithelial cells and how this might contribute to the prevention of pathogen adhesion, we pre-incubated gut epithelial Caco-2 cells with different hMOs and NDCs and subsequently exposed them to different bacterial pathogens. Anti-adhesion effects were tested for the gut pathogens *E. coli* ET8, *E. coli* LMG5862, *E. coli* O119, *E. coli* WA321, *Salmonella enterica* subsp. *enterica* LMG07233, and a lung pathogen *Klebsiella pneumoniae* LMG20218. They were tested after harvesting from both log and stationary growth phases. Effects of hMOs and NDCs as well as of the pathogens were tested on the gene expression in Caco-2 cells of galectin-1 (*LGALS1*), galectin-9 (*LGALS9*), glyocalyx-related molecules (glypican 1 [*GPC1*], hyaluronan synthase 3 [*HAS3*], exostosin glycosyltransferase 1 [*EXT1*], *EXT2*), *ICAM-1*, chemokine CXC motif ligands (*CXCL1*, *CXCL2*, *CXCL3* and *CCL20*), and antimicrobial peptide human defensin  $\beta$ -1 (*DEFB1*). We tested the effects of the most abundant hMOs in mother's milk, *i.e.* 2'-fucosyllactose (2'-FL) and 3-FL. Also, we tested and compared the effects of NDCs which are often used as substitutes in infant formula including inulins with different degrees of polymerization (DP3–DP10, DP10–DP60 and DP30–60) and pectins with different degrees of methylation (DM7, DM55 and DM69).

## 2. Materials and methods

### 2.1. Carbohydrates

The hMOs 2'-FL and 3-FL were obtained from Elicityl (France). Chicory based inulins with different degrees of polymerization,

*i.e.* highly soluble Frutafit® CLR inulin of DP3–DP10 and moderately soluble Frutafit® TEX! inulins of DP10–DP60 and DP30–DP60, were provided by Sensus (the Netherlands). Lemon pectins with different degrees of methylation including DM7, DM55, and DM69 were from CP Kelco (Denmark). All carbohydrates were dissolved with antibiotic-free cell culture medium into 2 mg ml<sup>-1</sup>.

### 2.2. Cell culture

Human intestinal epithelial Caco-2 cells (HTB-37, ATCC) at passage number 15–20 were cultured in Dulbecco's modified Eagle's medium (Lonza, Verviers, Belgium), supplemented with 10% (v/v, heat deactivated) fetal calf serum (Thermo Scientific, Breda, the Netherlands), 2.5% (v/v) HEPES, 50 U ml<sup>-1</sup> penicillin, 50 µg ml<sup>-1</sup> streptomycin, and 1% (v/v) non-essential amino acid (Sigma-Aldrich, Zwijndrecht, the Netherlands). The cells were routinely cultured in a 5% CO<sub>2</sub> incubator at 37 °C. To ensure the confluency and consistency of Caco-2 cells, the transepithelial electrical resistance (TEER) value was determined in transwell plates using an electric cell substrate impedance sensing (ECIS) system (Applied Biophysics). A total of  $1.5 \times 10^4$  cells per well were seeded into the 24-well plates and cultured for 21 days until a TEER value of  $300 \pm 10 \Omega \text{ cm}^2$  was reached.

### 2.3. Enteric pathogens and culture

Enteric pathogens including *Escherichia (E.) coli* ET8, *E. coli* LMG5862, *E. coli* O119, *E. coli* WA321, *Salmonella (S.) enterica* subsp. *enterica* LMG07233, and *Klebsiella (K.) pneumoniae* LMG20218 (ATCC) were tested. Bacterial strains were recovered from glycerol stocks in brain heart infusion (BHI) broth. After recovery, all strains were plated on BHI agar plates. A single colony from the agar plates was inoculated to BHI broth for overnight culture at 37 °C, and sub-cultured until either the logarithmic (log) phase or the stationary phase was reached. The cultures were centrifuged at 3000g for 10 min and the pellets were resuspended in PBS. The optical density (OD) value was adjusted to OD<sub>600</sub> =  $0.6 \pm 0.02$  (approx.  $1 \times 10^9$  CFU ml<sup>-1</sup>) and the pellets were washed once in PBS before inoculation.

### 2.4. Anti-adhesion assay

Caco-2 cells were pre-incubated with carbohydrates (2 mg ml<sup>-1</sup>) for 2 hours. The same volume of prepared pathogens from the log or stationary phase was inoculated for another 1 hour (*E. coli* LMG5862) or 2 hours (all other strains) of incubation. *E. coli* LMG5862 needed 1 hour incubation, because longer infection times destroyed the integrity of the Caco-2 cell monolayer. Cells pre-incubated without carbohydrates served as the control. After infection, Caco-2 cells were gently washed three times with PBS to remove the non-adherent bacteria. The adherent bacteria were released with 200 µl of 0.1% Triton X-100 for 10–15 min and subjected to serial dilutions. The drop-plate method was applied for quantification.<sup>27</sup> The results were expressed as relative adhesion compared to the controls.



## 2.5. RNA isolation and reverse transcription

The results of the anti-adhesion assays led to selection of 2'-FL, inulins, and pectins for gene expression analysis. As described above in the anti-adhesion assay, after 21 days of culture, Caco-2 cells were pre-incubated with 2'-FL, inulins, and pectins for 2 h. After infection with or without the pathogens, Caco-2 monolayers were lysed with TRIzol reagent (Life Technologies, Carlsbad, USA). Total RNA isolation was performed according to the manufacturer's instructions. SuperScript II reverse transcriptase (Invitrogen, Breda, the Netherlands) was used to synthesize cDNA for performing quantitative PCR.

## 2.6. Gene expression

*LGALS1*, *LGALS9*, *HAS3*, *EXT1*, *EXT2*, *GPC1*, *ICAM1*, *CXCL1*, *CXCL2*, *CXCL3*, *CCL20*, and *DEFB1* expressions were quantified using SYBR® Green Real-time PCR Master Mix (Roche, Basel, Switzerland). Reactions were carried out in 384-well PCR plates (Thermo Scientific, UK) using the ViiA7 Real-Time PCR system (Applied Biosystems). Expression levels were normalized to the housekeeping gene *GAPDH*. The  $2^{-\Delta\Delta Ct}$  method was used for the calculation of the fold changes in gene expression *versus* the group without pathogen infection. All the primers were synthesized by Sigma-Aldrich as listed in Table 1.<sup>28–36</sup>

## 2.7. Statistical analysis

All data were analyzed with GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, USA). The Kolmogorov–Smirnov test was performed to confirm the normal distribution of the data. The results were expressed as mean  $\pm$  SD of at least five experiments. All the data were analyzed using Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparison test. Significant difference was defined as  $p < 0.05$  ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  and  $****p < 0.0001$ ).  $p < 0.1$  was defined as a trend.<sup>37</sup>

## 3. Results

HMOs and NDCs have been suggested to modulate gut epithelial cell membrane bound receptors,<sup>38,39</sup> and by that prevent pathogen infections. It is unknown which and how these molecules can modulate gut epithelial cells such as Caco-2 and which pathogens might be prevented from binding to these cells. To this end, we tested the anti-adhesion effects of the hMOs 2'-FL and 3-FL, inulins with DP3–DP10, DP10–DP60, and DP30–DP60, and pectins with DM7, DM55, and DM69. We tested the adhesion of the enteric pathogens *E. coli* ET8, *E. coli* LMG5862, *E. coli* O119, *E. coli* WA321, and *S. enterica* subsp. *enterica* LMG07233. One lung pathogen *K. pneumoniae* LMG20218 was tested to determine the gut pathogen-specific effects. Pathogens from both log and stationary phases were tested as the bacteria–host interaction may differ with the bacterial growth phase. We pre-incubated hMOs and NDCs with Caco-2 cells before pathogen infection to determine possible prevention of pathogen adhesion after modulation of the intestinal epithelial cells by the molecules.

### 3.1. 2'-FL induced anti-adhesion effects on the pathogens from the log phase

**Log phase.** We pre-incubated Caco-2 cells with 2'-FL and 3-FL for 2 h before they were infected with the pathogens harvested from the log phase (Fig. 1A–F). We observed a 23.05% inhibition of *E. coli* O119 adherence to Caco-2 cells with 2'-FL ( $p < 0.05$ , Fig. 1C). Pre-incubating Caco-2 cells with 3-FL had no effect on the adhesion of the tested pathogens to Caco-2 cells.

**Stationary phase.** Pre-incubating Caco-2 cells with hMOs did not show any inhibition of adhesion of the tested pathogens from the stationary growth phase (Fig. 1G–L).

### 3.2. Inulins induced anti-adhesion effects on pathogens from both log and stationary phases in a DP dependent manner

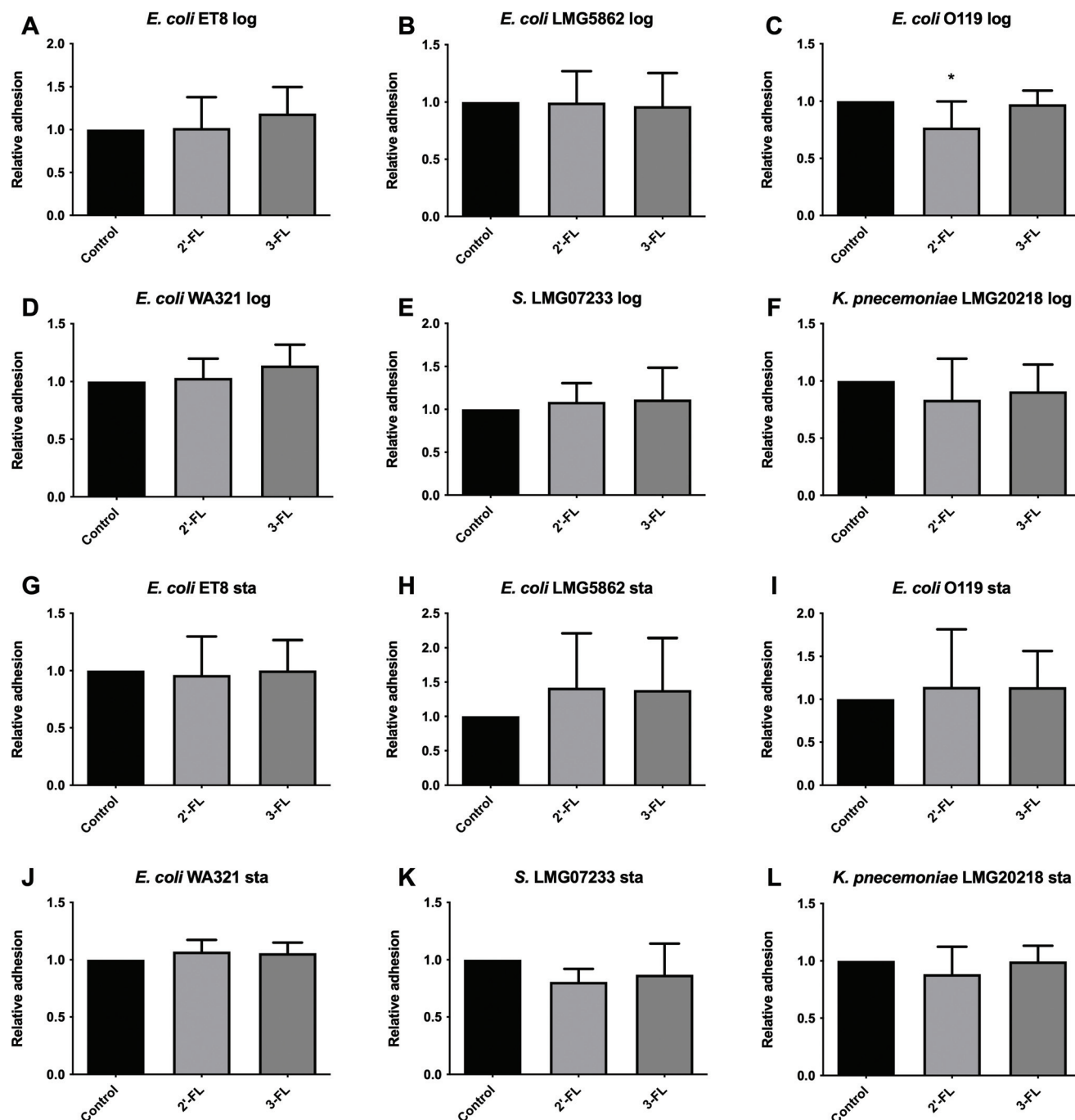
**Log phase.** Pre-incubating Caco-2 cells with inulins resulted in anti-adhesion effects on specific pathogens in a DP dependent

**Table 1** Primer sequences used for real-time qPCR<sup>28–36</sup>

Primer <sup>a</sup>	Forward	Reverse
<i>LGALS1</i>	5'-TGCAACAGCAAGGACGGC	5'-CACCTCTGCAACACTTCCA
<i>LGALS9</i>	5'-CTTTCATCACCACCATTTCTG	5'-ATGTGGAACCTCTGAGCACTG
<i>HAS3</i>	5'-TATACCGCGCGCTCCAA	5'-GCCACTCCGGAAGTAAGACT
<i>EXT1</i>	5'-CATAGGCGATGAGAGATTGT	5'-CAAGAATTGTGTCTGCTGTC
<i>EXT2</i>	5'-GGCTACGATGTCAGCATTCCTG	5'-GGCTTCTAGGTCCTCTCTGTAC
<i>GPC1</i>	5'-TATTGCCGAATGTGCTCAAGGGC	5'-ATGACACTCTCCACACCCGATGTA
<i>ICAM1</i>	5'-GGCCTCAGTCAGTGTGA	5'-AACCCATTCAGCGTCA
<i>CXCL1</i>	5'-AGTCATAGCCACACTCAAGAATGG	5'-GATGCAGGATTGAGGCAAGC
<i>CXCL2</i>	5'-CTCAAGAATGGGCAGAAAGC	5'-AAACACATTAGCGCAATCC
<i>CXCL3</i>	5'-GCAGGGAATTCACCTCAAGA	5'-GGTGCTCCCTTGTTCAGTA
<i>CCL20</i>	5'-GTGGCTTTTCTGGAATGGAA	5'-GACAAGTCCAGTGAGGCACA
<i>DEFB1</i>	5'-CCTTCTGCTGTTTACTCTCTGC	5'-GAATAGAGACATTGCCCTCCAC
<i>GAPDH</i>	5'-AAGATCATCAGCAATGCCTCTCTGC	5'-ATGGACTGTGGTCATGAGTCTTTC

<sup>a</sup> *LGALS1*, galectin 1; *LGALS9*, galectin 9; *HAS3*, hyaluronan synthase 3; *EXT*, exostosin glycosyltransferase; *GPC1*, glypican 1; *ICAM1*, intercellular adhesion molecule 1; *CXCL/CCL*, chemokine; *DEFB1*, defensin beta 1.





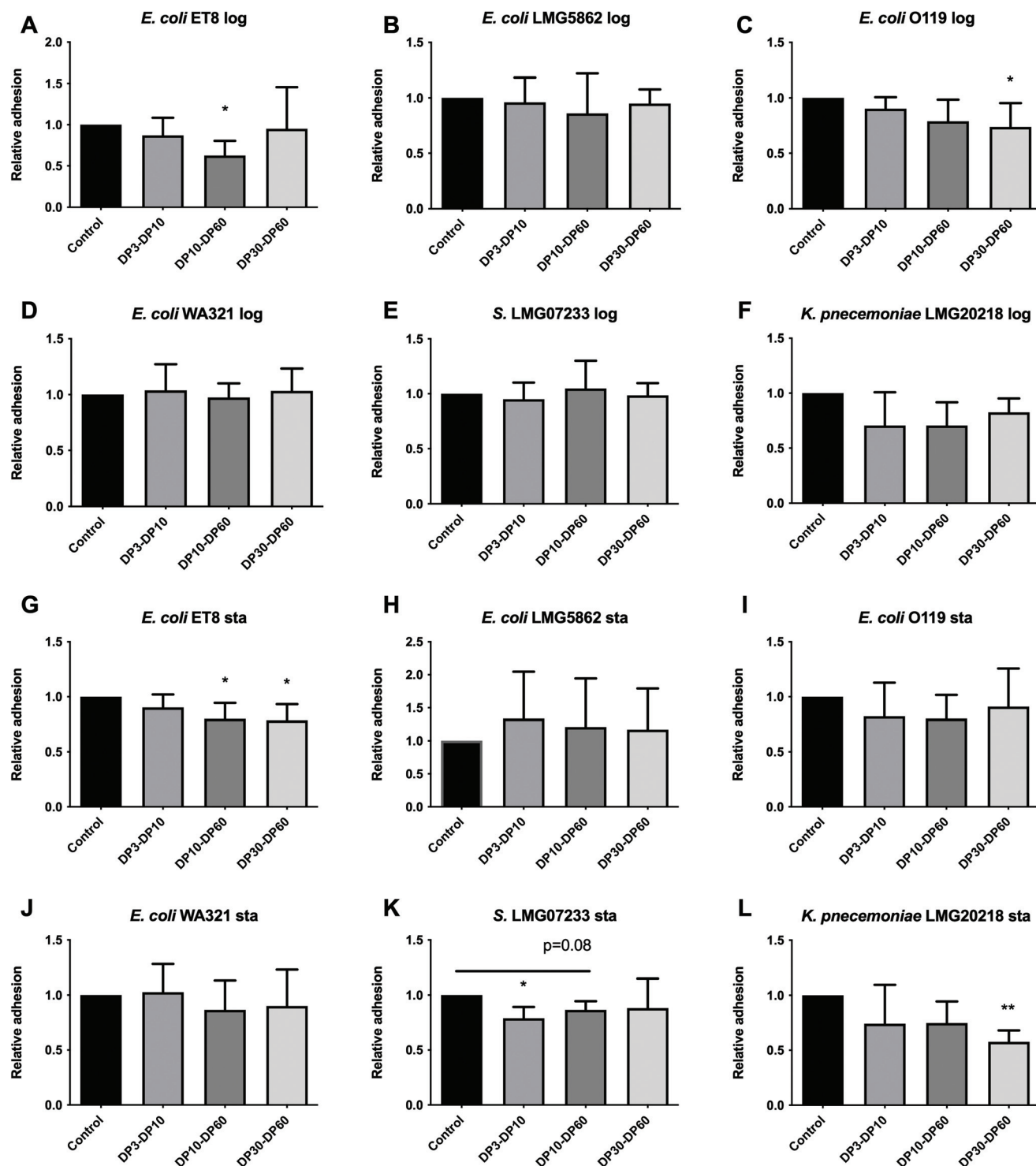
**Fig. 1** Anti-adhesion effects induced by hMOs on pathogens harvested from the log and stationary growth phases to Caco-2 cells. Caco-2 cells were cultured for 21 days until confluency. hMOs of 2'-FL and 3-FL were pre-incubated with Caco-2 cells for 2 h before infection with the pathogens harvested from the log phase. These were *E. coli* ET8 (A), *E. coli* LMG5862 (B), *E. coli* O119 (C), *E. coli* WA321 (D), *S. LMG07233* (E), and *K. pneumoniae* LMG20218 (F) or harvested from the stationary phase, i.e. *E. coli* ET8 (G), *E. coli* LMG5862 (H), *E. coli* O119 (I), *E. coli* WA321 (J), *S. LMG07233* (K), and *K. pneumoniae* LMG20218 (L) for another 2 h. After infection, the total colony forming units (CFUs) of the pathogens adhered to Caco-2 cells were determined by a drop-plating method. The group without hMOs served as the control. The adhesion of pathogens was normalized to the control (the control was set as 1). All data were expressed as mean  $\pm$  SD from five experiments. Statistical comparison was tested using one-way ANOVA (\* $p < 0.05$ ). Log and Sta stand for logarithmic and stationary stages of pathogen growth, respectively.

manner (Fig. 2A-F). DP10-DP60 inulin significantly reduced the adhesion of *E. coli* ET8 by 37.40% ( $p < 0.05$ , Fig. 2A), and DP30-DP60 inulin significantly reduced the adhesion of *E. coli*

O119 in the log phase by 26.25% ( $p < 0.05$ , Fig. 2C). DP3-DP10 and DP10-DP60 inulins reduced the adhesion of *K. pneumoniae* LMG20218 (Fig. 2F) by 29.44% and 29.45%,







**Fig. 2** Anti-adhesion effects induced by inulins on pathogens harvested from the log and stationary growth phases to Caco-2 cells. Caco-2 cells were cultured for 21 days until confluency. Inulins of DP3–DP10, DP10–DP60, and DP30–DP60 were pre-incubated with Caco-2 cells for 2 h before infection with the pathogens harvested from the log phase, i.e. *E. coli* ET8 (A), *E. coli* LMG5862 (B), *E. coli* O119 (C), *E. coli* WA321 (D), *S. LMG07233* (E), and *K. pneumoniae* LMG20218 (F) or harvested from the stationary phase, i.e. *E. coli* ET8 (G), *E. coli* LMG5862 (H), *E. coli* O119 (I), *E. coli* WA321 (J), *S. LMG07233* (K), and *K. pneumoniae* LMG20218 (L) for another 2 h. After infection, the total colony forming units (CFUs) of the pathogens adhered to Caco-2 cells were determined by a drop-plating method. The group without inulins served as the control. The adhesion of pathogens was normalized to the control (the control was set as 1). All data were expressed as mean  $\pm$  SD from five experiments. Statistical comparison was tested using one-way ANOVA (\* $p < 0.05$  and  $p < 0.1$  were considered a trend). Log and Sta stand for logarithmic and stationary stages of the pathogen growth, respectively.



respectively, but this did not reach statistical significance. Other pathogens were not influenced by the pre-incubation of inulins with Caco-2 cells.

**Stationary phase.** Pre-incubating Caco-2 cells with inulins induced anti-adhesion effects in the stationary phase also in a DP dependent manner. DP3–DP10 inulin selectively inhibited the adhesion of *S. enterica* subsp. *enterica* LMG07233 by 21.01% ( $p < 0.05$ , Fig. 2K). DP10–DP60 inulin significantly reduced the adhesion of *E. coli* ET8 in the stationary phase by 19.91% ( $p < 0.05$ , Fig. 2G) and had the tendency to inhibit the adhesion of *S. enterica* subsp. *enterica* LMG07233 (Fig. 2K) by 13.59%. DP30–DP60 inulin had anti-adhesion effects on both *E. coli* ET8 ( $p < 0.05$ , Fig. 2G) and *K. pneumoniae* LMG20218 ( $p < 0.01$ , Fig. 2L), which were 21.44% inhibition for *E. coli* ET8 and 42.34% for *K. pneumoniae* LMG20218.

### 3.3. Pectins induced anti-adhesion effects on the pathogens from both log and stationary phases in a DM dependent manner, and DM55 and DM69 pectins specifically increased the adhesion of *E. coli* WA321 from the stationary phase

**Log phase.** Pre-incubating Caco-2 cells with pectins had a DM dependent anti-adhesive effect on the pathogens. DM7 pectin significantly inhibited the adhesion of *E. coli* O119 ( $p < 0.01$ , Fig. 3C) and *K. pneumoniae* LMG20218 ( $p < 0.05$ , Fig. 3F) by 35.74% and 38.96%, respectively. DM55 pectin significantly reduced the adhesion of *E. coli* ET8 ( $p < 0.01$ , Fig. 3A) by 47.35%, *E. coli* LMG5862 ( $p < 0.01$ , Fig. 3B) by 43.41%, and *K. pneumoniae* LMG20218 ( $p < 0.05$ , Fig. 3F) by 43.37%. DM69 pectin selectively suppressed the adhesion of *E. coli* LMG5862 ( $p < 0.05$ , Fig. 3B) to Caco-2 cells by 35.96%.

**Stationary phase.** Pre-incubating Caco-2 cells with DM55 pectin significantly reduced the adhesion of *E. coli* ET8 by 40.59% ( $p < 0.01$ , Fig. 3G). In contrast, DM55 and DM69 pectins significantly increased the adhesion of *E. coli* WA321 ( $p < 0.05$ , Fig. 3J) by 92.58% and 84.83%, respectively. All of the tested pectins significantly inhibited the adhesion of the lung pathogen *K. pneumoniae* LMG20218 to Caco-2 cells, with a reduction of 34.71% by DM7 pectin ( $p < 0.05$ , Fig. 3L), with 54.99% by DM55 pectin ( $p < 0.01$ , Fig. 3L), and with 42.52% by DM69 pectin ( $p < 0.05$ , Fig. 3L).

### 3.4. 2'-FL and pectins but not inulins modulated the cell membrane glycosylation genes and inflammation associated genes in intestinal epithelial Caco-2 cells

As we found that 2'-FL, inulins, and pectins differently influenced the adhesion of pathogens to Caco-2 cells, we next determined the gene expression of cell membrane bound components that might be involved in pathogen adhesion and infection. We included the pathogens from either the log or the stationary phase whose adhesion to Caco-2 cells was significantly influenced. To this end, the expressions of cell membrane glycosylation genes, such as those of galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*), and inflammation associated genes, such as those of cell adhesion, chemotaxis target (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) and anti-microbial peptide (*DEFB1*), were studied.

**3.4.1. *E. coli* ET8. Log phase.** *E. coli* ET8 from the log phase did not influence the gene expressions of *LGALS1*, *LGALS9*, *EXT1*, *EXT2*, and *GPC1* (Fig. 4A, B, D–F), but significantly downregulated *HAS3* (Fig. 4C,  $p < 0.01$ ), which decreased by 34.25% compared to Caco-2 cells without infection. *E. coli* ET8 infection significantly upregulated adhesion and the chemotaxis target genes, i.e. *CXCL2* and *CCL20*, and showed a tendency to upregulate *CXCL1* and *CXCL3*, with an increase of 1.65-fold (Fig. 4I,  $p < 0.05$ ), 2.25-fold (Fig. 4K,  $p < 0.01$ ), 2.17-fold (Fig. 4H,  $p = 0.060$ ), and 2.92-fold (Fig. 4J,  $p = 0.066$ ), respectively. However, pre-incubating the cells with either DP10–DP60 inulin or DM55 pectin did not influence the above genes with *E. coli* ET8 infection.

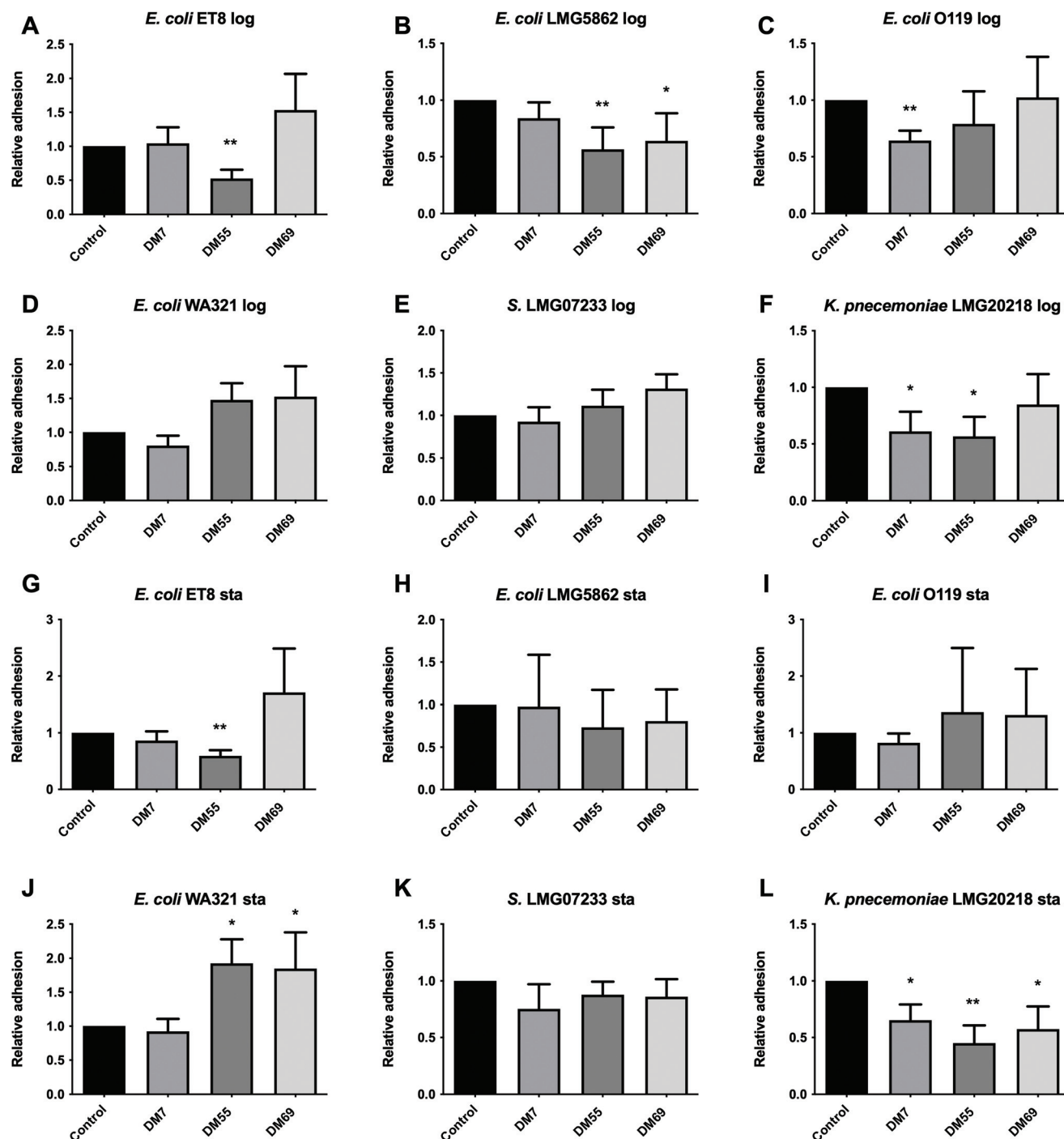
**Stationary phase.** *E. coli* ET8 from the stationary phase also significantly downregulated the glycosylation gene *HAS3*, with a reduction of 36.86% (Fig. 5C,  $p < 0.01$ ), but not *LGALS1*, *LGALS9*, *EXT1*, *EXT2*, and *GPC1* in Caco-2 cells. Pre-incubating the cells with DP10–DP60 inulin, DP30–DP60 inulin, or DM55 pectin did not influence the above genes with *E. coli* ET8 infection.

**3.4.2. *E. coli* LMG5862. Log phase.** With the infection of *E. coli* LMG5862 from the log phase, glycosylation genes including *HAS3*, *EXT1*, *EXT2*, and *GPC1* were significantly downregulated, with a reduction of 58.54% (Fig. 6C,  $P < 0.0001$ ), 48.94% (Fig. 6D,  $P < 0.0001$ ), 37.56% (Fig. 6E,  $P < 0.01$ ), and 54.47% (Fig. 6F,  $P < 0.0001$ ), respectively. Adhesion and chemotaxis target genes and the anti-microbial peptide gene were not influenced with *E. coli* LMG5862 infection. Pre-incubating Caco-2 cells with DM69 pectin but not DM55 pectin significantly upregulated the glycosylation gene *HAS3* (Fig. 6C,  $p < 0.05$ ), with an increase of 20.67%, compared to the cells exposed to *E. coli* LMG5862. Adhesion and chemotaxis target genes including *ICAM1*, *CXCL1*, *CXCL3*, and *CCL20* were also significantly upregulated by DM69 pectin, with a 1.09-fold (Fig. 6G,  $p < 0.0001$ ), a 79.47-fold (Fig. 6H,  $p < 0.01$ ), a 47.87-fold (Fig. 6J,  $p < 0.01$ ), and a 41.26-fold (Fig. 6K,  $p < 0.0001$ ) increase, respectively.

**3.4.3. *E. coli* O119. Log phase.** Similar to what we observed in *E. coli* LMG5862 (Fig. 6), *E. coli* O119 from the log phase also significantly downregulated the glycosylation gene *HAS3*, and showed a tendency to downregulate *GPC1*, with a reduction of 42.20% (Fig. 7C,  $P < 0.001$ ) and 25.56% (Fig. 7F,  $P = 0.099$ ), respectively. *E. coli* O119 infection significantly upregulated the adhesion and chemotaxis target genes of *ICAM1*, *CXCL1*, and *CCL20*, and a tendency with *CXCL3*, which was as high as 1.71-fold (Fig. 7G,  $p < 0.05$ ), 29.84-fold (Fig. 7H,  $p < 0.05$ ), 9.33-fold (Fig. 7K,  $p < 0.05$ ), and 23.59-fold (Fig. 7J,  $p = 0.066$ ) higher than the gene expression without *E. coli* O119 infection, respectively. Pre-incubating the cells with 2'-FL specifically significantly downregulated the expression of *ICAM1* (Fig. 7G,  $p < 0.05$ ). However, pre-incubating the cells with DP30–DP60 inulin or DM7 pectin did not influence the above genes with *E. coli* O119 infection.

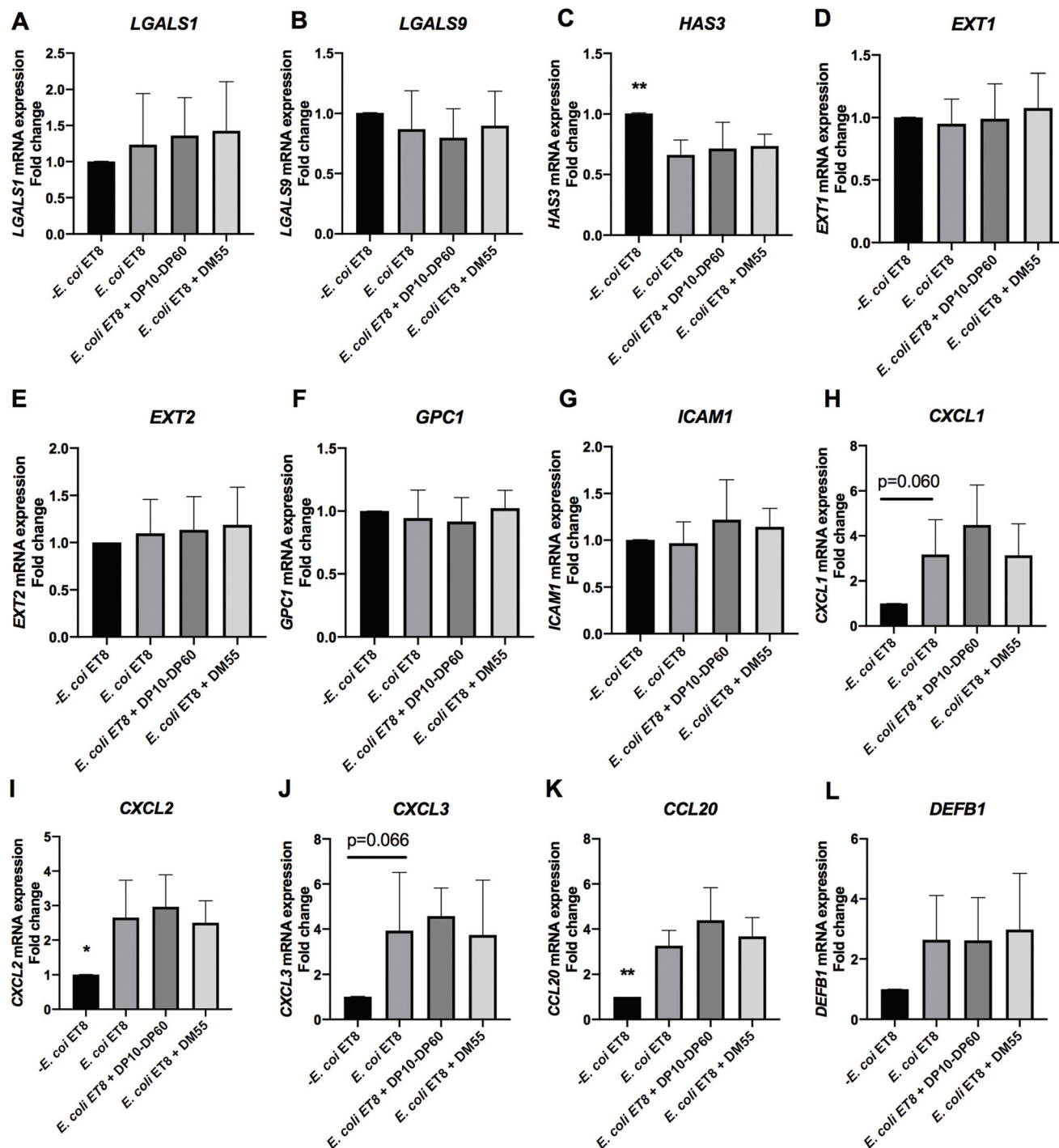
**3.4.4. *E. coli* WA321. Stationary phase.** *E. coli* WA321 from the stationary phase showed a tendency to downregulate the glycosylation gene *HAS3*, with a reduction of 41.87% (Fig. 8C,  $p = 0.099$ ). DM69 pectin was observed to have a tendency to upregulate *HAS3* (Fig. 8C,  $p = 0.071$ ). DM69 pectin dramatically upregulated all of the adhesion and chemotaxis target genes of *ICAM1*, *CXCL1*, *CXCL2*, *CXCL3*, and *CCL20*, with an increase of up to





**Fig. 3** Anti-adhesion effects induced by pectins on pathogens harvested from the log and stationary growth phases to Caco-2 cells. Caco-2 cells were cultured for 21 days until confluency. Pectins of DM7, DM55, and DM69 were pre-incubated with Caco-2 cells for 2 h before infection with the pathogens harvested from the log phase, i.e. *E. coli* ET8 (A), *E. coli* LMG5862 (B), *E. coli* O119 (C), *E. coli* WA321 (D), *S. LMG07233* (E), and *K. pneumoniae* LMG20218 (F) or harvested from the stationary phase, i.e. *E. coli* ET8 (G), *E. coli* LMG5862 (H), *E. coli* O119 (I), *E. coli* WA321 (J), *S. LMG07233* (K), and *K. pneumoniae* LMG20218 (L) for another 2 h. After infection, the total colony forming units (CFUs) of the pathogens adhered to Caco-2 cells were determined by a drop-plating method. The group without pectins served as the control. The adhesion of pathogens was normalized to the control (the control was set as 1). All data were expressed as mean  $\pm$  SD from five experiments. Statistical comparison was tested using one-way ANOVA (\* $p$  < 0.05 and \*\* $p$  < 0.01). Log and Sta stand for logarithmic and stationary stages of pathogen growth, respectively.

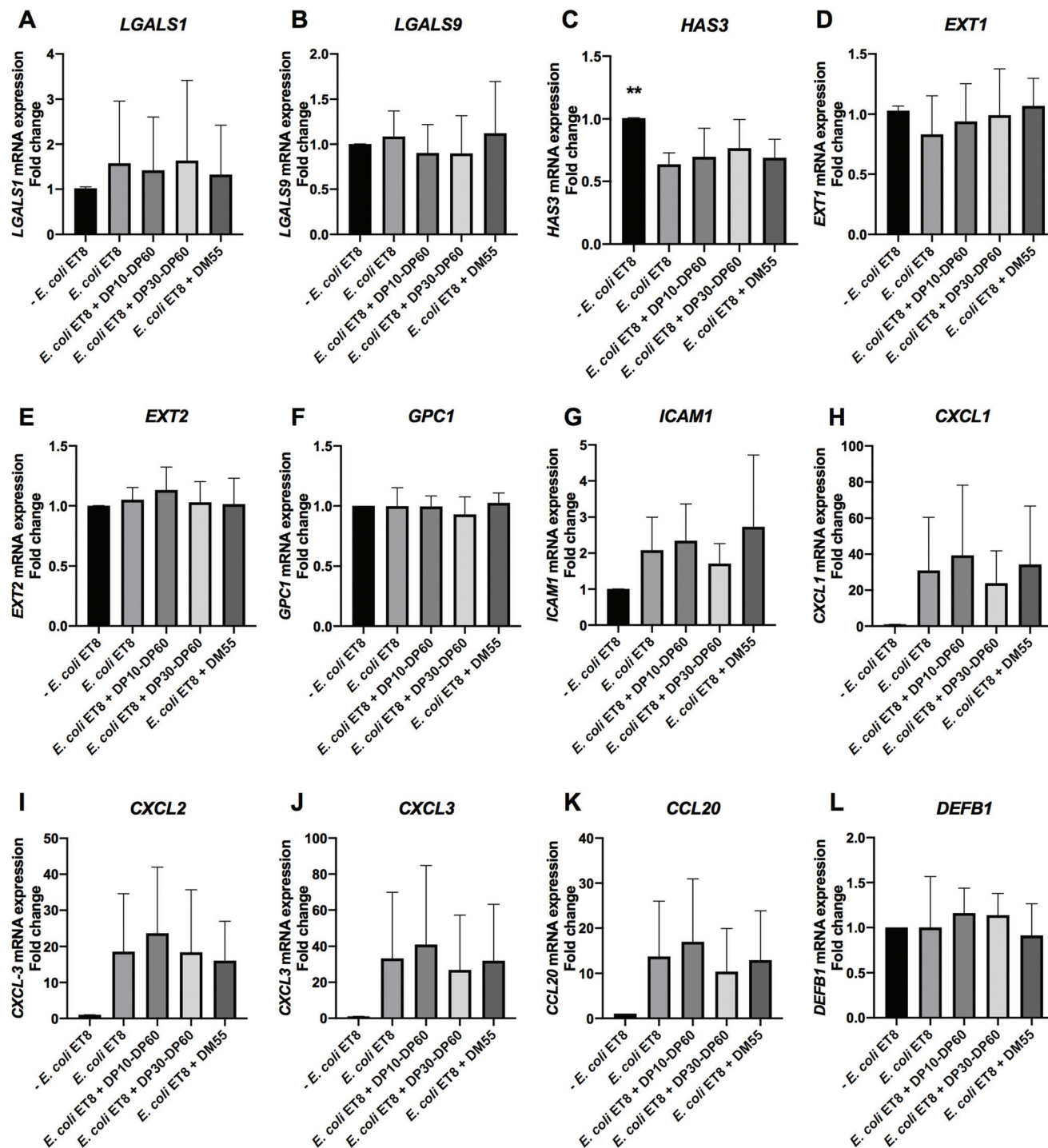




**Fig. 4** *E. coli* ET8 infection and the effects of DP10–DP60 inulin and DM55 pectin on cell membrane glycosylation and inflammation genes in the intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with DP10–DP60 inulin and DM55 pectin for 2 h before infection with *E. coli* ET8 harvested from the log phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) and the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results are presented as a fold change compared to the group without *E. coli* ET8 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *E. coli* ET8 infection, the group without *E. coli* ET8 infection and the group with pre-incubation of carbohydrates prior to *E. coli* ET8 infection (\* $p < 0.05$ , \*\* $p < 0.01$  and  $p < 0.1$  were defined as a statistical trend).

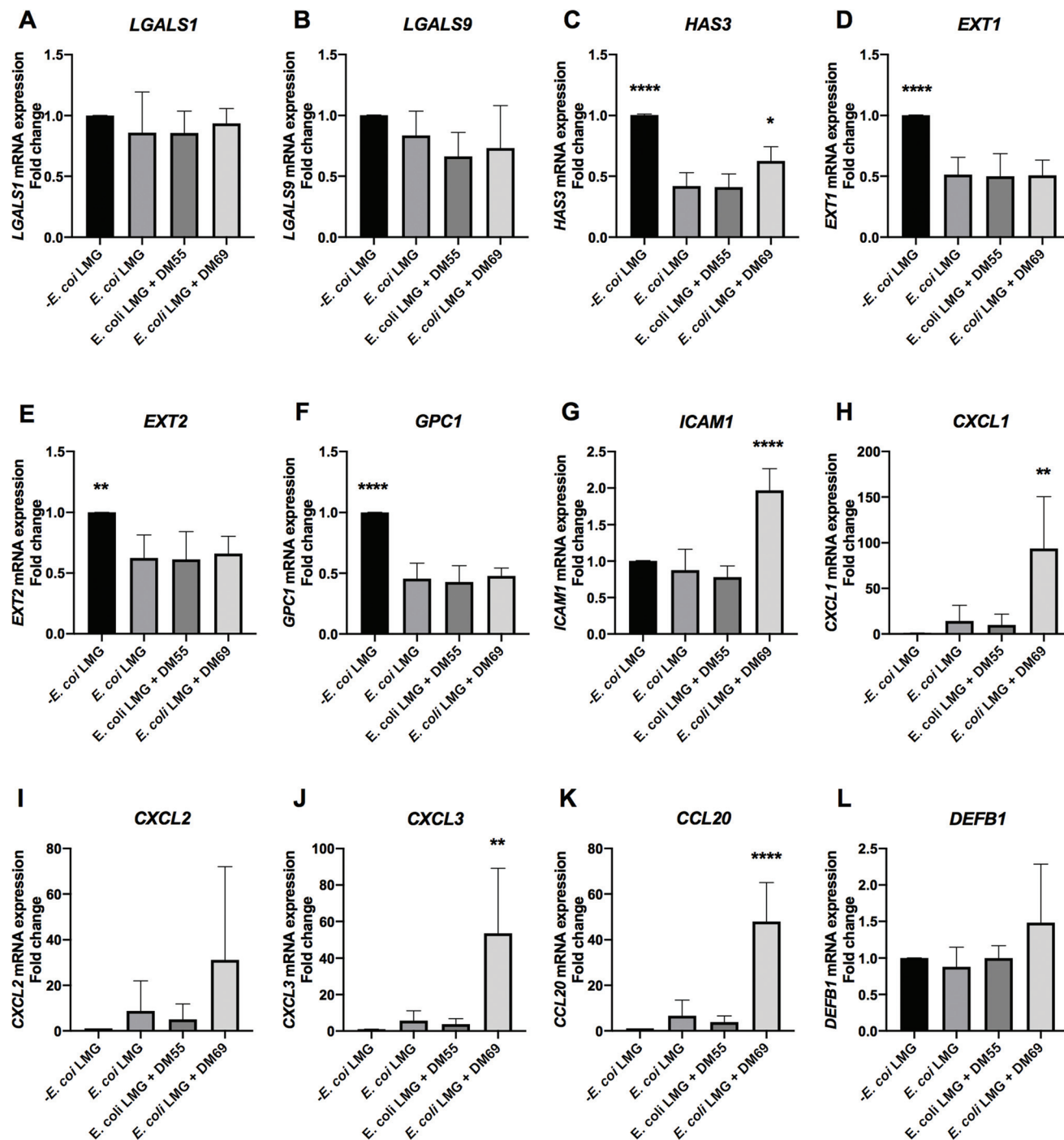






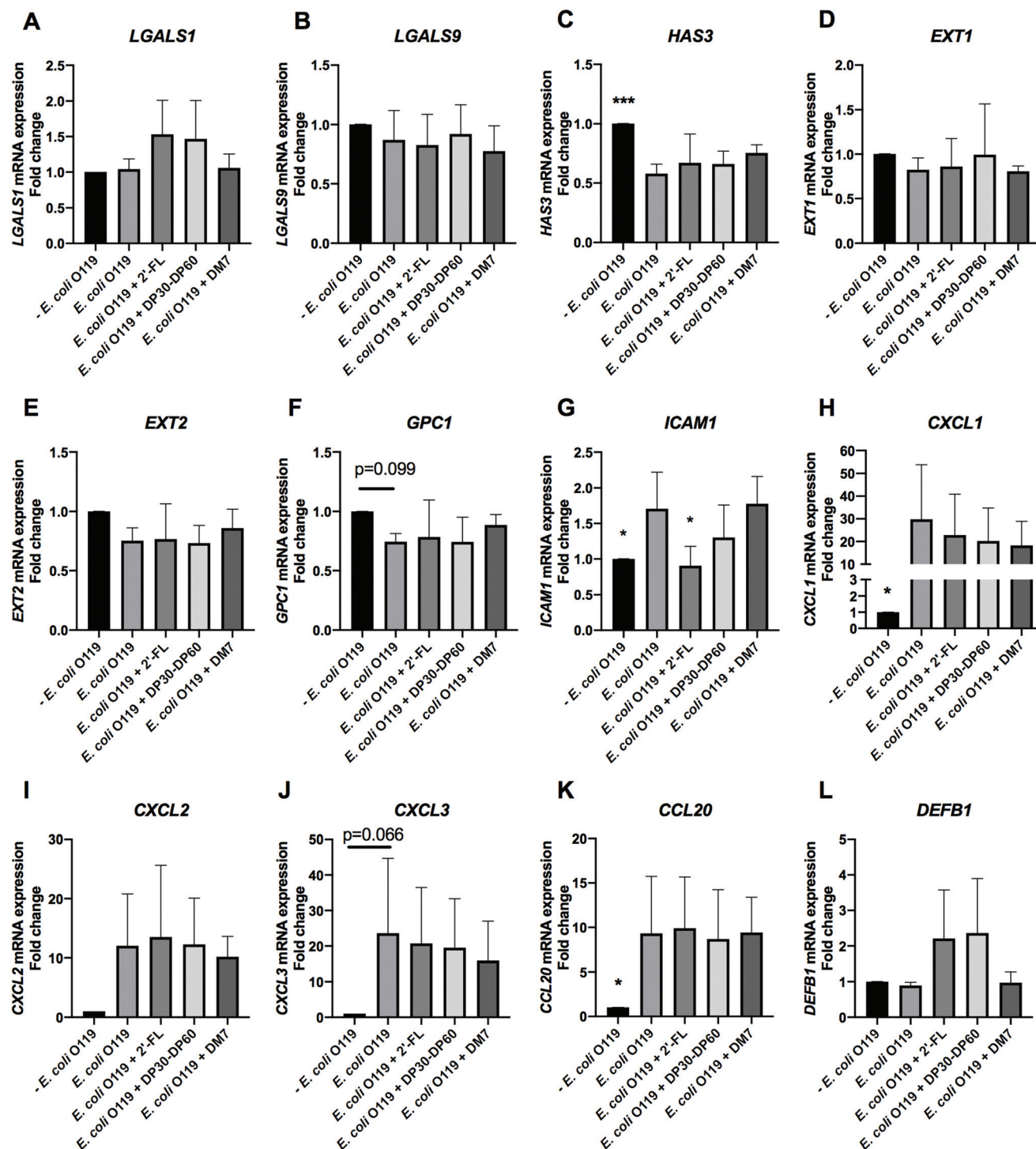
**Fig. 5** *E. coli* ET8 infection and the effects of DP10–DP60 and DP30–DP60 inulins and DM55 pectin on the cell membrane glycosylation and inflammation genes in intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with DP10–DP60 and DP30–DP60 inulins and DM55 pectin for 2 h before infection with *E. coli* ET8 harvested from the stationary phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) as well as the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results are presented as a fold change compared to the group without *E. coli* ET8 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *E. coli* ET8 infection, the group without *E. coli* ET8 infection and the group with pre-incubation of carbohydrates prior to *E. coli* ET8 infection (\*\* $p < 0.01$ ).





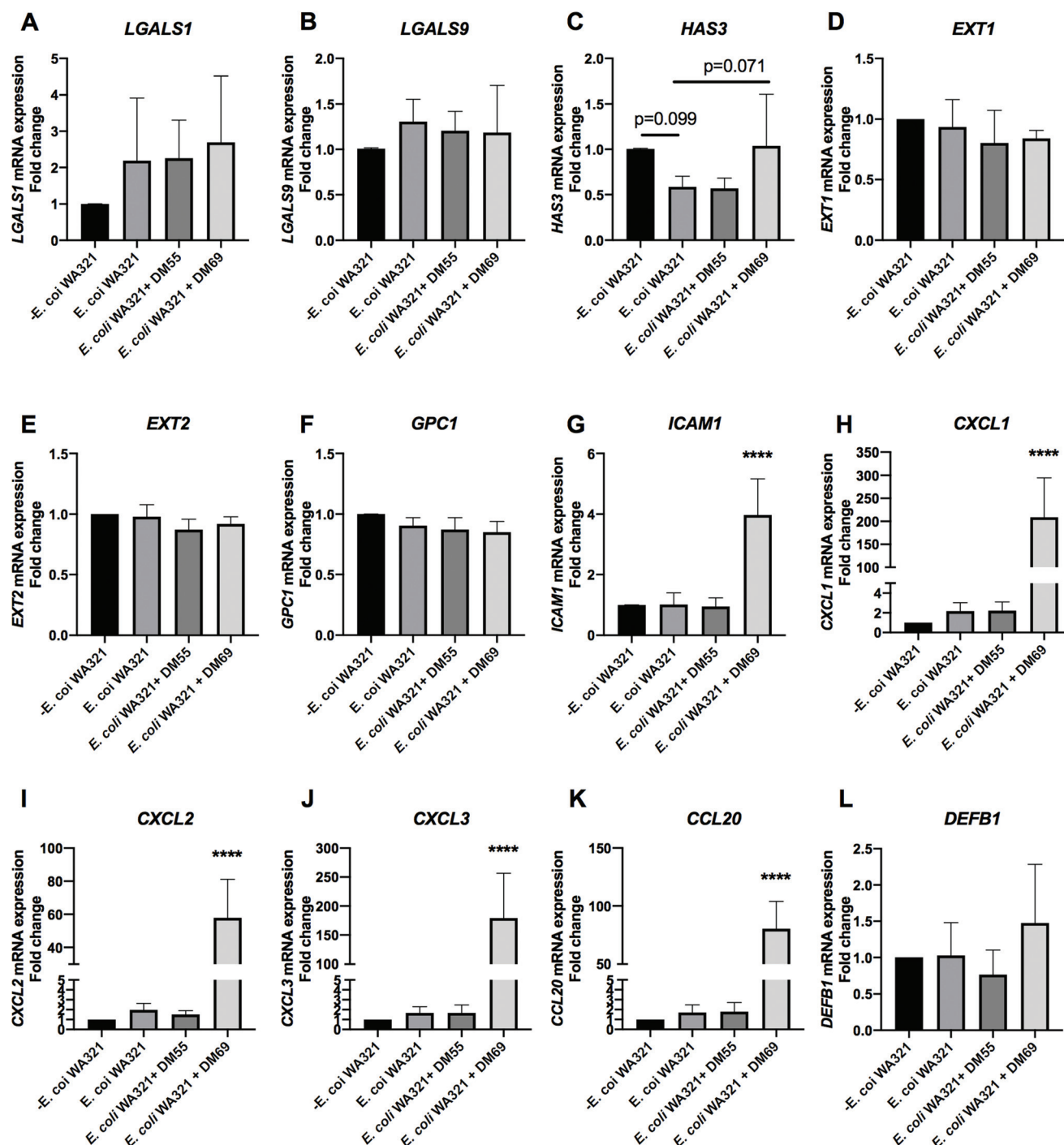
**Fig. 6** *E. coli* LMG5862 infection and the effects of DM55 and DM69 pectins on the cell membrane glycosylation and inflammation genes in intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with DM55 and DM69 pectins for 2 h before infection with *E. coli* LMG5862 harvested from the log phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycolyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) as well as the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results are presented as a fold change compared to the group without *E. coli* LMG5862 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *E. coli* LMG5862 infection, the group without *E. coli* LMG5862 infection and the group with pre-incubation of carbohydrates prior to *E. coli* LMG5862 infection (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$ ).





**Fig. 7** *E. coli* O119 infection and the effects of 2'-FL, DP30–DP60 inulin, and DM7 pectin on the cell membrane glycosylation and inflammation genes in intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with 2'-FL, DP30–DP60 inulin, and DM7 pectin for 2 h before infection with *E. coli* O119 harvested from the log phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) as well as the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results are presented as a fold change compared to the group without *E. coli* O119 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *E. coli* O119 infection, the group without *E. coli* O119 infection and the group with pre-incubation of carbohydrates prior to *E. coli* O119 infection (\* $p < 0.05$ , \*\*\* $p < 0.001$  and  $p < 0.1$  were considered a statistical trend).

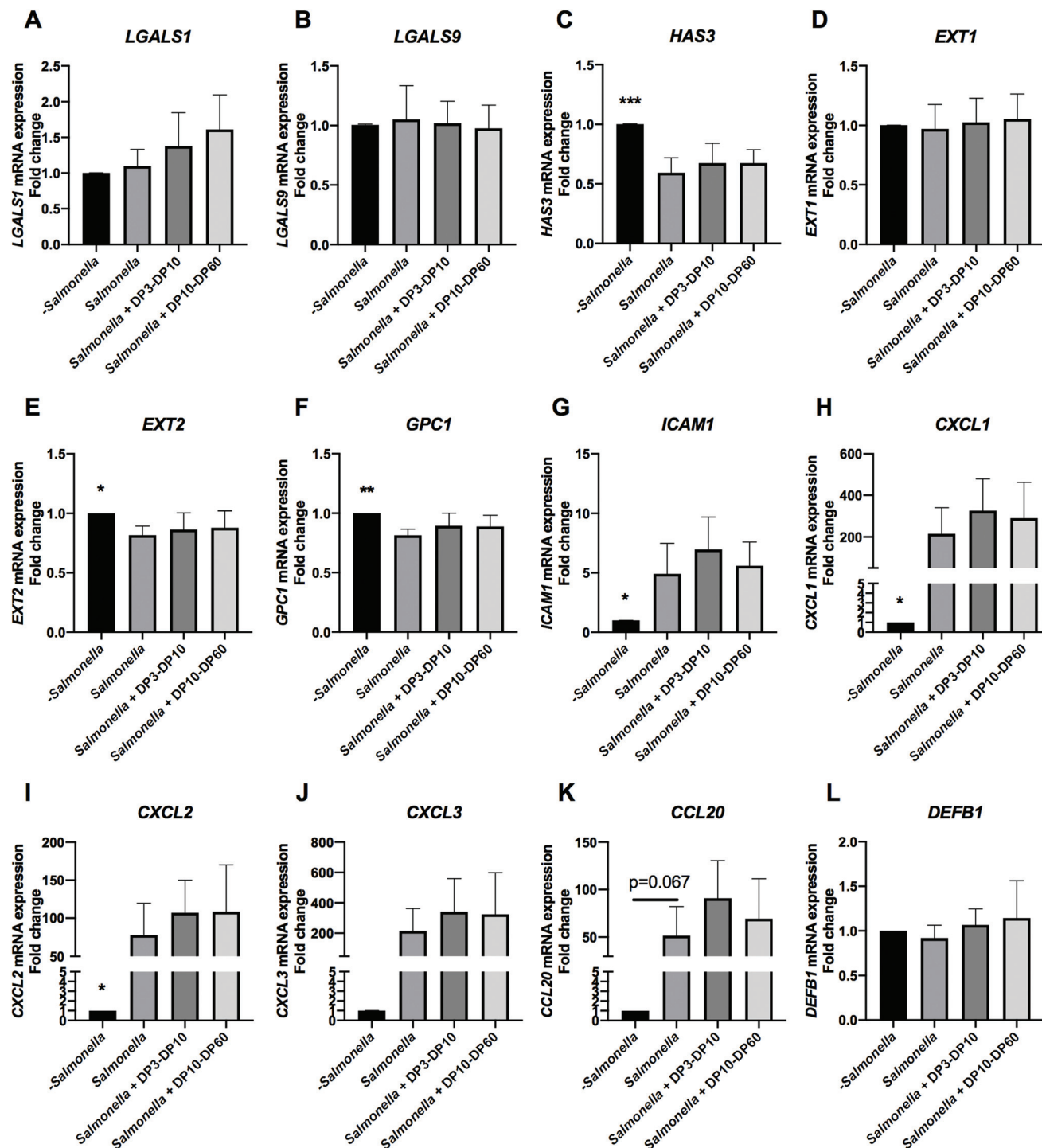




**Fig. 8** *E. coli* WA321 infection and the effects of DM55 and DM69 pectins on the cell membrane glycosylation and inflammation genes in intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with DM55 and DM69 pectins for 2 h before infection with *E. coli* WA321 harvested from the stationary phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) as well as the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results are presented as a fold change compared to the group without *E. coli* WA321 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *E. coli* WA321 infection, the group without *E. coli* WA321 infection and the group with pre-incubation of carbohydrates prior to *E. coli* WA321 infection (\*\*\*\* $p < 0.0001$  and  $p < 0.1$  were considered a statistical trend).







**Fig. 9** *S. enterica* subsp. *enterica* LMG07233 infection and the effects of DP3–DP10 and DP10–DP60 pectins on the cell membrane glycosylation and inflammation genes in intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with DP3–DP10 and DP10–DP60 inulin for 2 h before infection with *S. enterica* subsp. *enterica* LMG07233 harvested from the stationary phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) as well as the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results are presented as a fold change compared to the group without *S. enterica* subsp. *enterica* LMG07233 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *S. enterica* subsp. *enterica* LMG07233 infection, the group without *S. enterica* subsp. *enterica* LMG07233 infection and the group with pre-incubation of carbohydrates prior to *S. enterica* subsp. *enterica* LMG07233 infection (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and  $p < 0.1$  were considered a statistical trend).



3.97-fold (Fig. 8G,  $p < 0.0001$ ), 208.97-fold (Fig. 8H,  $p < 0.0001$ ), 57.88-fold (Fig. 8I,  $p < 0.0001$ ), 179.11-fold (Fig. 8J,  $p < 0.0001$ ), and 78.66-fold (Fig. 8K,  $p < 0.0001$ ), respectively. This upregulatory effect of DM69 pectin was not achieved by DM55 pectin.

**3.4.5. *S. enterica* subsp. *enterica* LMG07233. Stationary phase.** *S. enterica* subsp. *enterica* LMG07233 from the stationary phase significantly downregulated the glycosylation genes including *HAS3*, *EXT2*, and *GPC1* in Caco-2 cells, with a reduction of 40.89% (Fig. 9C,  $p < 0.001$ ), 18.31% (Fig. 9E,  $p < 0.05$ ), and 18.59% (Fig. 9F,  $p < 0.01$ ), respectively. The adhesion and chemotaxis target genes *ICAM1*, *CXCL1*, and *CXCL2* were significantly upregulated, with an increase of 3.92-fold (Fig. 9G,  $p < 0.05$ ), 214.26-fold (Fig. 9H,  $p < 0.05$ ), and 76.73-fold (Fig. 9I,  $p < 0.05$ ), respectively. *S. enterica* subsp. *enterica* LMG07233 showed a tendency to upregulate *CCL20* by 50.53-fold (Fig. 9K,  $p = 0.067$ ). *DEFB1* was not influenced by the infection of *S. enterica* subsp. *enterica* LMG07233. DP3–DP10 and DP10–DP60 inulins had no effect on the above genes.

**3.4.6. *K. pneumoniae* LMG20218. Log phase.** Consistently, *K. pneumoniae* LMG20218 from the log phase significantly downregulated the glycosylation genes *HAS3*, *EXT1*, and *GPC1*, and reduced it by 54.32% (Fig. 10C,  $p < 0.0001$ ), 37.25% (Fig. 10D,  $p < 0.0001$ ), and 20.54% (Fig. 10F,  $p < 0.05$ ), respectively. Among the adhesion and chemotaxis target genes, only *ICAM1* was significantly downregulated by 33.51% (Fig. 10G,  $p < 0.01$ ). *DEFB1* was not influenced by *K. pneumoniae* LMG20218. Pre-incubating Caco-2 cells with DM7 pectin significantly upregulated *HAS3* and *EXT1* by 9.62% (Fig. 10C,  $p < 0.05$ ) and 12.68% (Fig. 10D,  $p < 0.05$ ), respectively. *ICAM1* was significantly upregulated after pre-incubating Caco-2 cells with DM7 and DM55 pectins, with an increase of 38.29% (Fig. 10G,  $p < 0.001$ ) and 25.26% (Fig. 10G,  $p < 0.05$ ), respectively.

**Stationary phase.** *K. pneumoniae* LMG20218 from the stationary phase significantly downregulated *HAS3* by 40.15% (Fig. 11C,  $p < 0.0001$ ). Pre-incubating Caco-2 cells with carbohydrates resulted in pronounced effects as observed after *K. pneumoniae* LMG20218 exposure. The glycosylation genes of *LGALS1* and *HAS3* were significantly upregulated by DM69 pectin with an increase of 34.52% (Fig. 11A,  $p < 0.05$ ) and 15.69% (Fig. 11C,  $p < 0.05$ ), respectively. However, DM55 pectin significantly downregulated *LGALS9* and *GPC1* by 21.84% (Fig. 11B,  $p < 0.05$ ) and 16.75% (Fig. 11F,  $p < 0.05$ ), respectively. DM7 pectin also significantly downregulated *GPC1* by 14.62% (Fig. 11F,  $p < 0.05$ ). The adhesion and chemotaxis target genes *ICAM1*, *CXCL1*, *CXCL2*, *CXCL3*, and *CCL20* were all significantly upregulated by DM69 pectin, with an increase of 2.35-fold (Fig. 11G,  $p < 0.0001$ ), 77.49-fold (Fig. 11H,  $p < 0.0001$ ), 27.51-fold (Fig. 11I,  $p < 0.01$ ), 47.03-fold (Fig. 11J,  $p < 0.0001$ ), and 67.19-fold (Fig. 11K,  $p < 0.0001$ ), respectively. DM69 pectin pre-incubation also significantly upregulated *DEFB1* by 79.45% (Fig. 11L,  $p < 0.01$ ).

## 4. Discussion

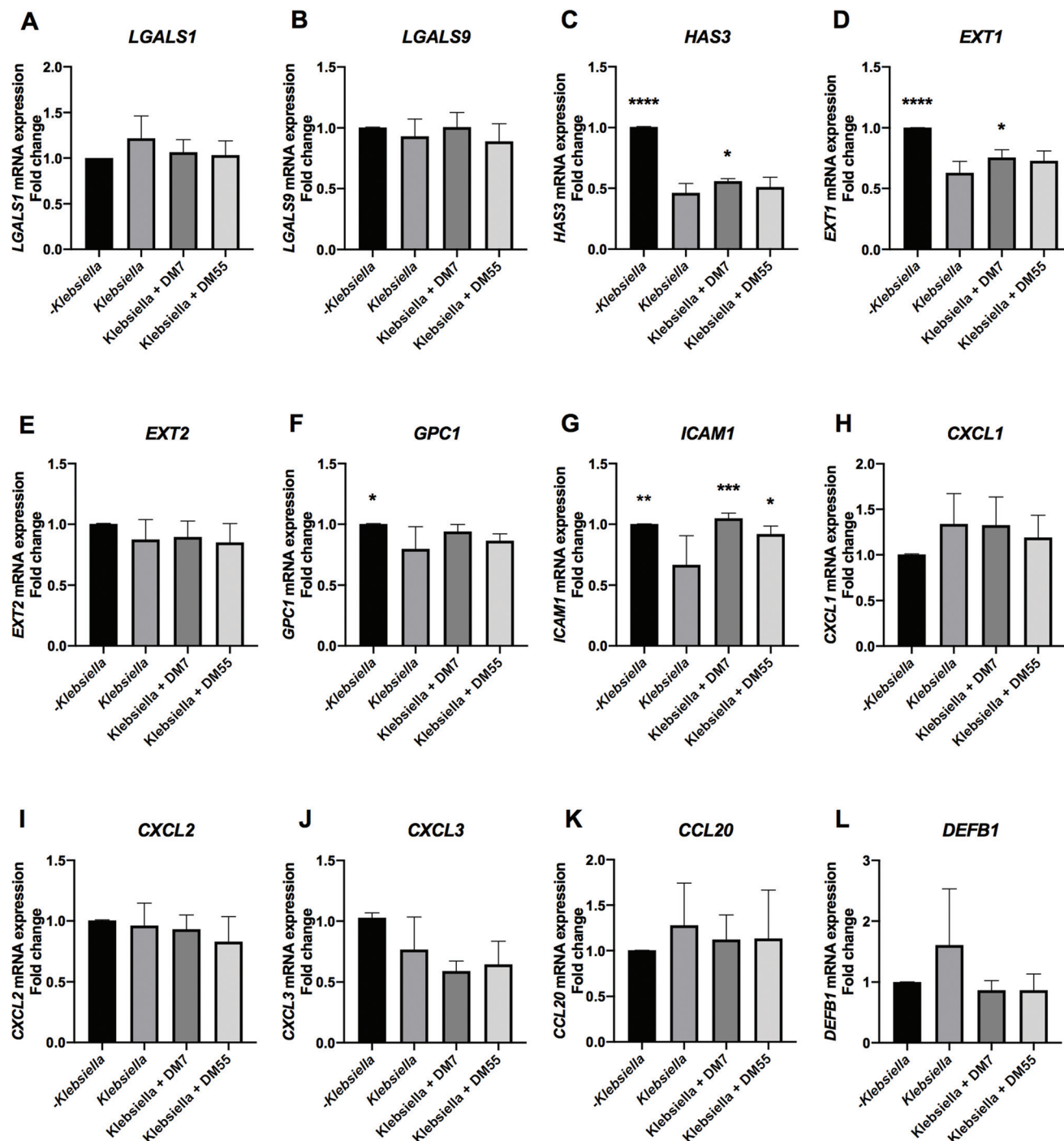
Previous studies have shown that hMOs and NDCs can modulate the gut epithelial cells and thus result in lower chances for

pathogen infection.<sup>40,41</sup> It is however largely unknown which mechanisms are responsible for the anti-adhesion effects of hMOs and NDCs and whether these are pathogen specific. This knowledge is of significant importance for the effective tailoring of infant formula to modulate gut epithelial cells to resist pathogen infections. This was one of the reasons to perform this study and we show that specific structures of hMOs, inulins and pectins induce anti-adhesion effects of specific pathogens from different growth phases. We show that these specific hMO and NDC structures modulate intestinal epithelial glycosylation genes and inflammation associated genes, which may explain the anti-adhesion effects of the hMO and NDC structures on these pathogens towards gut epithelial cells.

Our data show that pre-incubating Caco-2 cells with the hMO 2'-FL but not with 3-FL inhibit the adhesion of *E. coli* O119 from the log phase. The specific pathogen adhesion inhibitory effect of 2'-FL might be explained by the downregulation of the gene expression of intercellular adhesion molecule-1 (*ICAM1*) on Caco-2 cells. *ICAM1* is a trans-membrane receptor that is expressed in the intestine and involved in the inflammatory process induced by pathogens.<sup>18</sup> This may indicate that 2'-FL can directly influence the expression of the intestinal epithelial receptor *ICAM1*, which is probably essential for the adhesion of *E. coli* O119. Although both 2'-FL and 3-FL belong to the fucosylated hMO and have the same lactose core structure, it is probably the difference in fucosylation that should explain the difference in their biological effects.<sup>42</sup>

All the inulins tested in this study showed an inhibitory effect on the adhesion of pathogens to Caco-2 cells, but the magnitude of prevention of adhesion was shown to be dependent on the chain length of the inulins. The short chain inulin DP3–DP10 only inhibited the adhesion of *S. enterica* subsp. *enterica* LMG07233 from the stationary phase, while the long chain inulin DP10–DP60 and DP30–DP60 inulin induced a larger inhibitory effect on pathogen adhesion. The DP10–DP60 inulin inhibited the adhesion of *E. coli* ET8 from both log and stationary phases, and *S. enterica* subsp. *enterica* LMG07233 from the stationary phase. The DP30–DP60 inulin inhibited the gut pathogens of *E. coli* O119 from the log phase, *E. coli* ET8 from the stationary phase and one lung pathogen of *K. pneumoniae* LMG20218 from the stationary phase. The anti-adhesion effects by inulins were neither induced by the cell membrane glycosylation nor by the expressions of the tested inflammation associated genes. Other genes such as mucosal immunity and barrier function related genes might be involved in the anti-adhesion effects of inulins on the pathogens.<sup>43</sup> Effects of different inulins were also dependent on the growth phase of the pathogens. Pathogens in different growth phases contribute differently to the infection process.<sup>44</sup> For instance, *E. coli* was demonstrated to be more adhesive and infectious in the stationary phase than in the log phase.<sup>44</sup> Our data show that pre-incubating Caco-2 cells with especially the longer chain DP30–DP60 inulin inhibited the adhesion of *E. coli* ET8 from the stationary phase but not from the log phase, illustrating that inulins may have different efficacies on

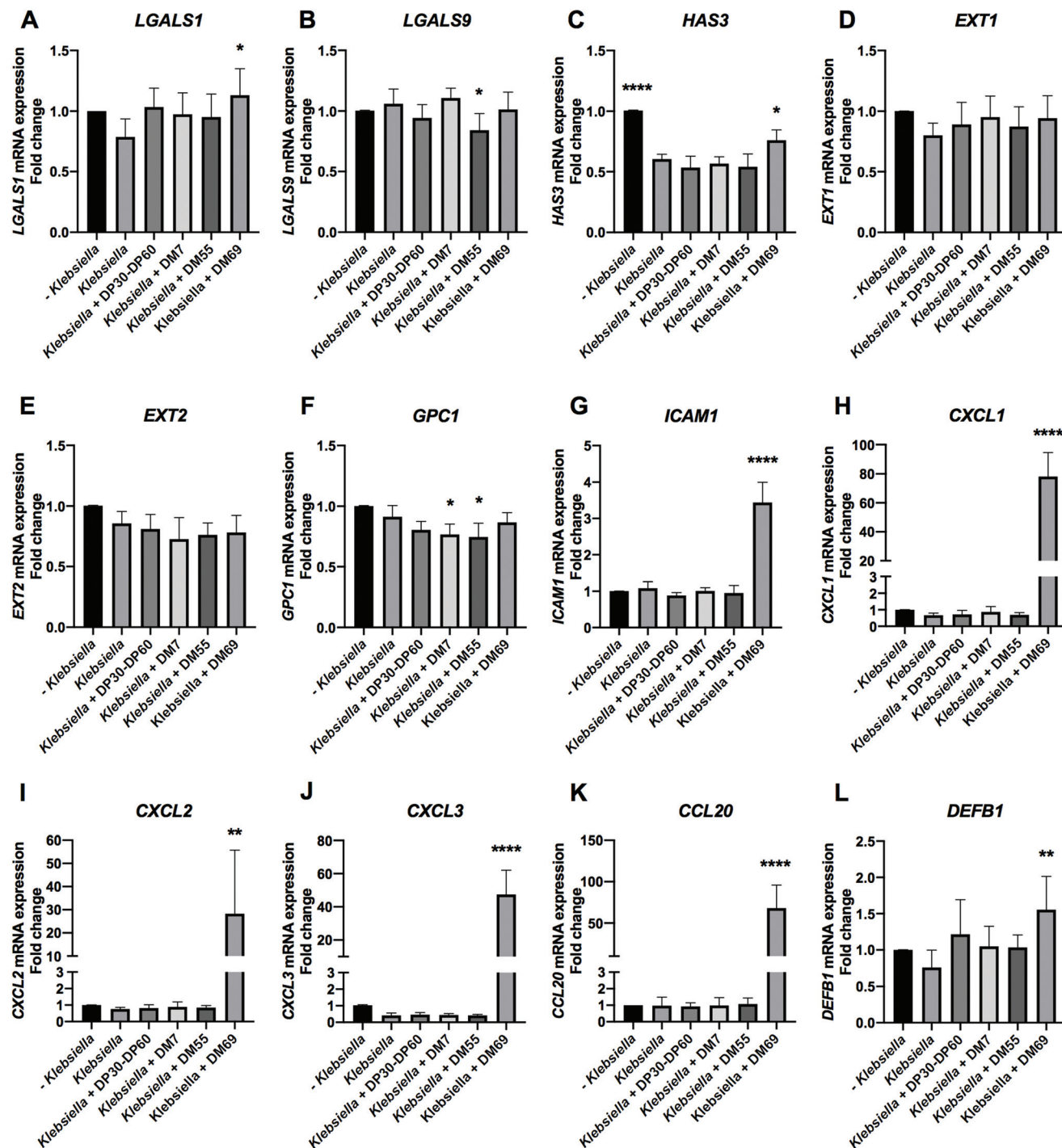




**Fig. 10** *K. pneumoniae* LMG20218 infection and the effects of DM7 and DM55 pectins on the cell membrane glycosylation and inflammation genes in intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with DM7 and DM55 pectins for 2 h before infection with *K. pneumoniae* LMG20218 harvested from the log phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) as well as the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results were presented as a fold change compared to the group without *K. pneumoniae* LMG20218 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *K. pneumoniae* LMG20218 infection, the group without *K. pneumoniae* LMG20218 infection and the group with pre-incubation of carbohydrates prior to *K. pneumoniae* LMG20218 infection (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ ).







**Fig. 11** *K. pneumoniae* LMG20218 infection and the effects of DP30–DP60 inulin and DM7, DM55, and DM69 pectins on the cell membrane glycosylation and inflammation genes in intestinal epithelial Caco-2 cells. Caco-2 cells were cultured for 21 days and pre-incubated with DP30–DP60 inulin and DM7, DM55, and DM69 pectins for 2 h before infection with *K. pneumoniae* LMG20218 harvested from the stationary phase. After infection, the cell membrane glycosylation genes [galectins (*LGALS1* and *LGALS9*) and glycocalyx (*HAS3*, *EXT1*, *EXT2* and *GPC1*)] and inflammation associated genes [cell adhesion and chemotaxis target genes (*ICAM1*, *CXCL1*, *CXCL2*, *CXCL3* and *CCL20*) as well as the anti-microbial peptide gene (*DEFB1*)] were quantified by evaluating the mRNA expression with real-time RT-PCR. The results are presented as a fold change compared to the group without *K. pneumoniae* LMG20218 infection. Statistical analysis was performed using one-way ANOVA, comparing to the group only with *K. pneumoniae* LMG20218 infection, the group without *K. pneumoniae* LMG20218 infection and the group with pre-incubation of carbohydrates prior to *K. pneumoniae* LMG20218 infection (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$ ).





pathogens from different growth phases.<sup>26</sup> This suggests that mixtures of inulins might be necessary to prevent pathogen infections in infant formula.

2'-FL and inulins are the most widely applied hMO and NDC in infant formula, and both belong to the family of neutral oligosaccharides. Although many health benefits have been shown in clinical trials for these molecules,<sup>45,46</sup> they still do not fully mimic the functions of hMOs in mother's milk that also contains acidic oligosaccharides.<sup>1</sup> It has been suggested that acidic oligosaccharides are important for specific health benefits.<sup>1</sup> Such an alternative acidic NDC substitute for hMO might be pectin that contains galacturonic acid and provides negatively charged carboxyl groups similar to what can be found on acidic sialylated hMO.<sup>1,47</sup> Here, pectins with DM7, DM55, and DM69 all modulated gut epithelial cells, such that both gut pathogens and the lung pathogen had much less ability to adhere to the epithelial cells. This may suggest that for the anti-pathogenic effects of pectins, their negatively charged carboxyl groups may be a valuable addition for infant formula.<sup>48</sup>

The DM55 and DM69 pectins may govern the anti-adhesion effects on pathogens through modulation of cell membrane glycosylation-associated galectins. Our data demonstrate that pre-incubating Caco-2 cells with both DM55 and DM69 pectins inhibited the adhesion of *K. pneumoniae* LMG20218 from the stationary phase, but differently modulated the cell membrane glycosylation-associated galectin genes *LGALS1* and *LGALS9*, which are responsible for the synthesis of galectin-1 and galectin-9, respectively. Galectins are a family of conserved glycan-binding proteins.<sup>14</sup> Galectin-1 is widely distributed on epithelial cells in the small intestine and is downregulated in celiac disease.<sup>14</sup> Here, we show pre-incubating Caco-2 cells with DM69 pectin increased *LGALS1* expression after the exposure of epithelial cells to *K. pneumoniae* LMG20218. This finding suggests a possible rescuing effect of DM69 pectin on the maintenance of gut homeostasis during infection.<sup>14</sup> Galectin-9 is different from galectin-1 and highly expressed in patients with food allergy and considered to be a target for preventing allergic diseases.<sup>16</sup> Here, we show that through pre-incubating Caco-2 cells with DM55 pectin, *LGALS9* was downregulated after exposure to *K. pneumoniae* LMG20218, which may suggest an inflammation suppressive effect of DM55 pectin.

The DM7, DM55, and DM69 pectins may also govern the anti-adhesion effects of pathogens through modulation of glycosylation-associated genes in epithelial cells. Pre-incubating Caco-2 cells with DM7, DM55, and DM69 pectins inhibited the adhesion of *K. pneumoniae* LMG20218 from the log phase (DM7 and DM55 pectins), *K. pneumoniae* LMG20218 from the stationary phase (DM7, DM55 and DM69 pectins), and *E. coli* LMG5862 from the log phase (DM69 pectin). This was associated with the downregulation of the epithelial glycosylation-associated genes including *HAS3*, *EXT1*, and *GPC1*. In particular, *HAS3*, the hyaluronic acid synthase gene, was significantly downregulated by all of the tested pathogenic strains and this was observed with pathogens from both log and stationary phases. HAS is responsible for the synthesis of HA, which is a

highly viscous component of the intestinal epithelial glycocalyx glycosaminoglycan side chain and is responsible for tissue repair and anti-inflammatory effects.<sup>49</sup> *HAS3* downregulation may lead to the lower production of HA, resulting in a thinner glycocalyx barrier, which may increase the chances for pathogen infection. However, DM7 and DM69 pectin pre-incubation significantly upregulated *HAS3*, which may enhance the barrier function of Caco-2 cells<sup>49</sup> and reduce pathogen adhesion as we demonstrate for *E. coli* LMG5862 from the log phase and *K. pneumoniae* LMG20218 from both log and stationary phases.

Another glycocalyx gene that was downregulated by the exposure to pathogens was *EXT1*. *EXT1* is involved in the elongation of one important glycocalyx molecule, which is the glycosaminoglycan side chain heparan sulfate (HS). HS supports the organogenesis, maintains gut barrier integrity, and can be cleaved by gut bacterial enzymes.<sup>50</sup> *EXT1* was downregulated in response to the exposure of *K. pneumoniae* LMG20218 from the log phase, but pre-incubating Caco-2 cells with DM7 pectin prevented this lowering of *EXT1* expression. Moreover, DM7 and DM69 pectins upregulated the glycosaminoglycan chains HA and HS. This was different for DM55 pectin, which did not influence HA and HS, but induced a downregulation of the protein backbone glypican 1 (*GPC1*). DM7 pectin also induced a downregulation of *GPC1*. *GPC1* acts as a carrier for the glycosaminoglycan chains in the glycocalyx layer.<sup>51</sup> Through the downregulation of *GPC1*, DM7 and DM55 pectins may modify the composition of the glycocalyx layer that provides binding sites for *K. pneumoniae* LMG20218. Consequently, DM7 and DM55 pectin pretreatment may reduce the adhesion of *K. pneumoniae* LMG20218 to epithelial cells, as we also found in our study. Overall, our data show that genes involved in glycocalyx synthesis are downregulated with pathogen infection, but specific pectins with DM7, DM55, and DM69 may prevent this by re-modeling the glycosaminoglycan side chains and the protein backbone of the glycocalyx and by that inhibit pathogen adhesion.

We observed a pathogen strain dependent effect on inflammation-associated genes and a structure dependent effect of pectins on restoring the expression of these genes. With the exception of *E. coli* LMG5862 and *E. coli* WA321, other tested gut pathogens induced upregulation of the cell adhesion and chemotaxis genes *ICAM1*, *CXCL1*, *CXCL2*, *CXCL3*, or *CCL20*. These genes were reported to be associated with Crohn's disease and ulcers.<sup>52</sup> Even though *E. coli* LMG5862 alone did not influence these genes in Caco-2 cells, DM69 pectin pre-incubation dramatically upregulated *ICAM1*, *CXCL1*, *CXCL3*, and *CCL20*, which may suggest that DM69 pectin induced the facilitation of epithelial responses to *E. coli* LMG5862. For *E. coli* WA321, an enhanced epithelial attachment was measured after DM69 pectin pre-incubation, suggesting that DM69 pectin may facilitate the adhesion of *E. coli* WA321 to Caco-2 cells. Effects of other pathogens on the expression of inflammation-associated genes were however not changed by pectin pre-incubation.

Different from the gut pathogens, the lung pathogen *K. pneumoniae* LMG20218 induced a lowering effect on inflam-



mation related genes. *K. pneumoniae* LMG20218 from the log phase downregulated *ICAM1*. A different interaction of *K. pneumoniae* LMG20218 with the host epithelium may suggest a different infection strategy compared to the gut pathogens mentioned above. This might be related to the fact that *K. pneumoniae* evolved with stronger resistance to antibiotic treatment, and a different biosynthesis and transportation pathway for virulence factors such as lipopolysaccharides.<sup>53</sup> Interestingly, pre-incubation with DM7 and DM55 pectins restored the downregulation of *ICAM1* induced by *K. pneumoniae* LMG20218 from the log phase. The upregulation of the inflammation genes might imply more production of pro-inflammatory cytokines and anti-microbial peptides,<sup>20,54</sup> which might lead to the lower *K. pneumoniae* LMG20218 adhesion to Caco-2 cells as we observed. This is also in line with the established assumption that pro-inflammatory signaling is crucial for *K. pneumoniae* LMG20218 clearance.<sup>55</sup>

## 5. Conclusions

In summary, we demonstrate that 2'-FL as well as pectins may prevent the adhesion of pathogens through modulating glycosylation genes of galectin and glycocalyx, and inflammatory genes in gut epithelial cells. The effects on this epithelium-pathogen interaction are structure-dependent. In particular, low DM pectin with DM7 have such an effect, while high DM pectins with DM55 and DM69 may also have a risk of modulating epithelial cells as such that it leads to increased adhesion of specific pathogens. Inulins probably exert anti-pathogenic effects through its well-known effect on enhancing barrier function in gut epithelial cells.<sup>43</sup> We also showed that pathogens themselves modulate gene expression in gut epithelial cells. Specific NDC molecules, *i.e.* DM69 pectin, upregulated inflammation-associated genes when gut epithelial cells were exposed to pathogens, which may suggest that DM69 pectin has an immune activating effect and by that contributes to the clearance of pathogen adhesion.<sup>55</sup> This information can be used in the design of tailored infant formula with anti-adhesion properties on pathogens.

## Author contributions

C.K. and P.d.V. conceived and designed the experiments. C.K., M.W., and B.J.d.H. performed the experiments. C.K. and B.J.d.H. analyzed the data. C.K., M.B., and P.d.V. wrote the paper.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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