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RESEARCH ARTICLE

Anti-Inflammatory Properties of Ripe and Unripe Papaya Pectin in a Neonatal Human Stem Cell-Derived Ileum Model

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ABSTRACT

Pectin, a dietary fiber found in fruits and vegetables, has recognized anti-inflammatory properties. While papaya is rich in pectin, the impact of different ripening stages on its anti-inflammatory effects within the human neonatal gastrointestinal tract remains unexplored. Ripe and unripe papaya contain structurally distinct pectins, potentially leading to differing anti-inflammatory effects. Herein, ripe and unripe papaya pectins were tested in an in vitro model of human neonatal intestinal mucosa, in the presence or absence of commensal *Escherichia coli* (*E. coli*) as a surrogate for early intestinal colonizers. Papaya pectin enhanced cell viability regardless of ripening stage or the presence of *E. coli*. Both pectins reduced the IL-6 production in cultures exposed to *E. coli*, but only unripe pectin decreased IL-8 levels. Additionally, treatment with either pectin downregulated genes related to tight junctions and innate immune signaling (e.g., *claudin-3*, *catenin-1*, *catenin-3*, *SUGT1*, *TRAF6*, and *NLRP3*) in the presence of *E. coli*. Dimensionality reduction analyses further suggest that pectin induced distinct transcriptional profiles depending on the presence of bacteria. These results demonstrate that papaya pectin modulates epithelial barrier in a manner dependent on ripening stage and microbial context, providing novel insights into its functional and stage-specific anti-inflammatory activity.

1 | Introduction

Dietary interventions are a promising complement to traditional pharmacological therapies for managing inflammatory gut diseases. Low-fiber diets are known to disrupt gut microbiota composition, promote the growth of mucus-degrading bacteria, and heighten susceptibility to intestinal inflammation [1]. Fiber pectins are complex polysaccharides found in various fruits and vegetables, known for their ability to modulate gut microbiota composition, enhance intestinal barrier function, and exert anti-inflammatory effects [2]. Studies in piglets have shown that pectin supplementation improves epithelial barrier integrity by modulating the bile acid pool [3], altering the composition of gut microbiota, and increasing the production of short-chain fatty acids (SCFAs), which are beneficial metabolites derived from microbial fermentation of dietary fiber [4, 5]. Pectins, particularly those derived from acidic oligosaccharides, are generally recognized as safe for infant consumption [6]. However, the limited availability of human-relevant in vitro models that accurately represent the neonatal and infant gastrointestinal tract limits our

Abbreviations: HDIM, human stem cell-derived polarized Intestinal monolayer Model.

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ability to comprehensively assess their biological effects. Pectin structure consists of a central backbone of galacturonic acid units (homogalacturonan), with branched regions containing neutral sugar side chains (rhamnogalacturonan I and II), such as arabinose and galactose [7].

Papaya (*Carica papaya* L.), a tropical fruit rich in dietary fiber pectin, has demonstrated various health benefits, including increased satiety, decreased cholesterol and glucose levels, and anti-inflammatory activities [8–10]. In contrast to citrus, apple, or passion fruit pectins, which undergo minimal modification in pectin structure or abundance during ripening, papaya exhibits a high metabolic profile, leading to rapid ripening and profound changes in pectin structure [11–13]. Additionally, unlike citrus and apple pectins, papaya uniquely combines highly soluble pectins with endogenous proteolytic enzymes. Among these, papain may contribute to its enhanced ability to modulate the infant gut microbiota and reduce inflammation [14]. However, to date, no studies have evaluated the effects of different papaya ripening stages on inflammatory responses within a human-relevant model of the neonatal gastrointestinal tract. Ripe and unripe fruits differ significantly in their physical properties, nutritional composition, and functional activities [11, 12]. Ripe fruits are softer, sweeter, and more vibrant in color due to the pectin degradation, the conversion of complex carbohydrates to simple sugars, and carotenoid accumulation. These changes are primarily driven by the activity of various endogenous enzymes, particularly polygalacturonases and β-galactosidase [11, 12].

In contrast, unripe fruits remain firmer, less sweet, and richer in chlorophyll but lower carotenoid content [12, 15]. Understanding how ripe and unripe papaya pectins modulate inflammatory responses within the human intestine is crucial for optimizing their therapeutic potential and informing dietary recommendations for individuals with inflammatory gut diseases. This information is particularly relevant to preterm infants at risk for necrotizing enterocolitis (NEC), a severe gastrointestinal disorder characterized by intestinal inflammation and necrosis. NEC affects approximately 7% of infants weighing less than 1500 g [16–19] and is associated with high morbidity and mortality rates [17, 20–22]. Our central hypothesis is that the ripening stage of papaya influences the effectiveness of its pectin in reducing neonatal intestinal inflammation, primarily through structural modifications that occur during ripening. To test this hypothesis, we used neonatal terminal ileum explants as a source of stem cells to build a human stem cell-derived polarized intestinal monolayer model (HDIM) that mimics the in vivo small intestine environment [23–25]. We also used the commensal *Escherichia coli* strain HS, originally isolated from a healthy human volunteer [26], which serves as a surrogate for early intestinal microbiota [27]. At birth, the intestinal microbiota comprises a collection of microorganisms, dominated by the commensal *E. coli*, known as the pioneer settlers [28–30].

2 | Experimental Section

2.1 | Human Tissues

Non-diseased residual terminal ileal tissues were collected from three neonates aged 4 days, 1 month, and 2 months during clinical care surgeries. These residual tissues were determined to be normal based on the surgeon's gross inspection. Specimens were de-identified and replaced with randomized codes consisting of numbers and letters. The only data retained from these specimens were the approximate collection date (month/year), sex, and age. A protocol outlining the collection and use of these specimens was submitted to the University of Maryland IRB, and a study exemption (not human research determination) was approved (HP-00082602). All tissues were processed within 1 h of collection.

2.2 | Stem Cell Isolation, Culture Media, and the Establishment of HDIM

HDIM was prepared using stem cells (SCs) isolated from crypts of the neonatal terminal ileum, as previously described [23–25]. Briefly, tissues were minced and digested with a collagenase solution for 30 min, with intermittent mixing. Crypts were isolated via low-speed centrifugation, resuspended in DMEM/F12 media, and counted under a microscope. SCs were co-cultured with γ-irradiated 3T3-J2 fibroblasts in Matrigel-coated 6-well plates with an expansion medium containing a cocktail of growth factors until semi-confluence. Dissociated SC clusters were seeded at a density of 0.5×10^6 cells on Matrigel-coated 0.4-µm transwells along with 0.2×10^6 γ-irradiated fibroblasts. Monolayers were grown to confluence and differentiated for 4 days using the air-liquid interface (ALI) technique. Differentiated epithelial cells in the HDIM were exposed to six experimental conditions: (1) DMEM/F12 alone as a negative control, (2) *E. coli* strain HS (40×10^6 bacteria/mL), (3) ripe papaya pectin alone, (4) unripe papaya pectin alone, (5) *E. coli* strain HS combined with ripe papaya pectin, and (6) *E. coli* strain HS combined with unripe papaya pectin. After 5 h of exposure, cells were preserved in RNALater for RNA extraction and stored at −80°C. Supernatants from the apical compartments were collected and stored at −20°C for subsequent analysis of cell viability and the production of pro-inflammatory IL-6 and IL-8 cytokines by ELISA [27, 31, 32].

2.3 | Pectin Preparation and Dosage Information

The pectins used in the study were derived from the water-soluble fraction (WSF) extracted from ripe and unripe papayas (*Carica papaya* L. cv. "Golden"), as previously described [33]. To remove endotoxins, the pectins were purified using a chromatography column packed with Polymyxin B-Agarose resin (P1411, Sigma, St. Louis, MO, USA). Endotoxin removal was confirmed using the Limulus amebocyte lysate (LAL) assay cartridges (Charles River Laboratories, Wilmington, MA, USA). The purified samples were then lyophilized and used in cell culture experiments at 0.3 mg/mL, a concentration previously determined to be non-cytotoxic in murine fibroblasts [22]. The physicochemical characteristics of the extracted pectins were previously reported. Briefly, ripe papaya pectin displayed a lower molecular weight (175 kDa) than unripe pectin (232 kDa). The degree of esterification was similar for both pectins. Monosaccharide composition analysis revealed higher glucose contents in ripe pectin, whereas unripe pectin contained greater proportions of galactose and arabinose [22].

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2.4 | Cell Viability

Cell viability was measured in HDIM supernatants using a commercial lactate dehydrogenase (LDH) kit (CytoTox 96, Promega, Madison, WI, USA), as previously described [31]. LDH is a stable cytosolic enzyme that is released into the cell culture supernatant upon cell lysis. The supernatants were harvested 5 h after stimulation and kept at −20°C until assayed. An LDH positive control was used to create a standard curve in triplicate with a 2-fold dilution, and the sample absorbance was interpolated to obtain the relative number of lysed cells. The LDH positive control top standard gives the same level of enzyme found in 13 500 lysed L929 fibroblasts.

2.5 | Cytokine Production

Levels of IL-6 and IL-8 were measured using commercial ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA). Supernatants were collected 5 h after the addition of pectin and/or *E. coli* to the cultures and stored at −20°C until analysis. Uninfected cells cultured with medium only served as negative controls. Cytokine concentrations were quantified in pg/mL using standard curves prepared in duplicate with lyophilized standards provided in the kits. The assay sensitivity for the cytokines ranged from 0.3 to 2.5 pg/mL.

2.6 | RT-PCR

Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and RNA concentrations were measured with a NanoDrop 1000 spectrophotometer. A total of 1.0 µg of RNA was treated with DNase I (Qiagen) and reverse transcribed using the RT^2 First Strand Reverse Transcription Kit (Qiagen). Amplified material was detected using RT^2 SYBR Green qPCR Mastermix (Qiagen). Real-time quantitative PCR was performed on an ABI 7900HT thermocycler (Applied Biosystems) under the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression of tight junction genes (*CLDN1* [Claudin 1], *CLDN3* [Claudin 3], *CLDN4* [Claudin 4], *CTNNA1* [Catenin 1], and *CTNNA3* [Catenin 3]) was calculated against the housekeeping gene *RPLP0* and normalized to the media-only negative control using the comparative $2^{(-\Delta\Delta Ct)}$ method [27]. In some experiments, Human Antibacterial RT^2 Profiler PCR Arrays (Qiagen) were used to assess the expression of 84 key genes involved in the innate immune response to bacteria. Arrays were performed according to the manufacturer's instructions, and results were analyzed using the GeneGlobe Data Analysis Center, a web-based software platform provided by Qiagen.

2.7 | Statistical Analysis

All statistical tests were performed using Prism software (version 10, GraphPad Software, La Jolla, CA, USA). Comparisons between groups were carried out using paired, two-tailed, mixed-effects models with the assumption of sphericity. Principal component analysis (PCA) was performed as described previously [34–36]. p values of <0.05 were considered statistically significant for comparisons between groups. Post hoc testing was carried out using Fisher's least significant difference (LSD) procedure. No correction for multiple comparisons was applied.

3 | Results

3.1 | Papaya Pectins Increase Cell Viability

Since avoiding toxicity is crucial in any treatment, we began our studies by investigating the impact of papaya pectins at different ripening stages on cell viability. We used an HDIM to mimic the in vivo small-intestine environment [23–25]. *E. coli* was also included as a surrogate commensal to represent the gut microbiota [27]. HDIM was exposed to *E. coli* in the presence or absence of ripe or unripe pectins at 0.3 mg/mL. HDIM cultures with media only or supplemented with either ripe or unripe pectins served as controls. After 5 h of exposure, cell viability was assessed using a commercial LDH assay kit [31]. Surprisingly, regardless of the type of pectin used (ripe or unripe) or the presence of *E. coli*, pectin treatments resulted in significantly lower levels of cell death compared to media-only controls (NC), as determined using paired, two-tailed mixed-effects models with no correction applied for multiple comparisons (n = 5 independent experiments with 2 replicates each) (Figure 1). These findings suggest that papaya pectins may play a protective role in neonatal intestinal health by enhancing cell viability in the presence of *E. coli*, a bacterial organism that forms part of the intestinal microbiota.

3.2 | Production of Pro-Inflammatory Cytokines After Papaya Pectin Exposure

Pectins are known for their anti-inflammatory properties [1]. Thus, we next measured the levels of production of pro-inflammatory cytokines, specifically interleukin (IL)-6 and IL-8, by ELISA. Supernatants from neonatal HDIM were collected 5 h after exposure to *E. coli* in the presence or absence of ripe and unripe papaya pectins. Uninfected cells cultured with medium only, or cells exposed to pectins without bacteria, served as controls. Interestingly, cultures exposed to *E. coli* and treated with either ripe or unripe pectins showed a significant reduction in IL-6 production compared to cultures exposed to *E. coli* alone (n = 5 independent experiments with 2 replicates each) (Figure 2A). Surprisingly, a decrease in IL-8 levels was observed only in *E. coli* cultures treated with unripe pectin (n = 5 independent experiments with 2 replicates each) (Figure 2B). This suggests a potential differential effect on pro-inflammatory cytokine levels depending on the ripening stage and cytokine evaluated.

3.3 | Effect of Pectins on Tight Junctions' Gene Expression Profile

Given that pectin treatment in the ileum HDIM significantly decreased IL-6 production, we examined the expression of tight junction genes implicated in maintaining epithelial barrier integrity. Previous research has demonstrated that IL-6 can influence intestinal permeability by upregulating the expression

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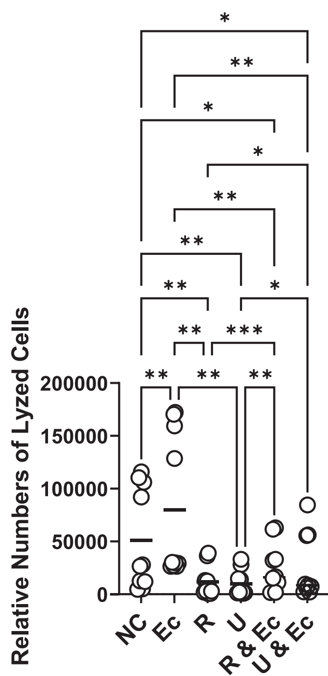

FIGURE 1 | Cell viability after pectin exposure. HDIM was left untreated (NC) or exposed to *E. coli* strain HS (Ec) in the absence or presence of 0.3 mg/mL of ripe or unripe pectin (R & Ec, U & Ec). Cells alone (NC) or in the presence of pectin only (R, U) were used as controls. After 5 h, supernatants were collected and used to measure cell viability using a commercial lactate dehydrogenase (LDH) assay. Data represents five independent experiments with two replicates each; horizontal lines represent the mean values of pooled data. Paired two-tailed mixed-effects models were used to compare the multiple groups. p values < 0.05 were considered statistically significant. The levels of significance are: *, 0.01–0.05; **, 0.001–0.01; ***, 0.0001–0.001.

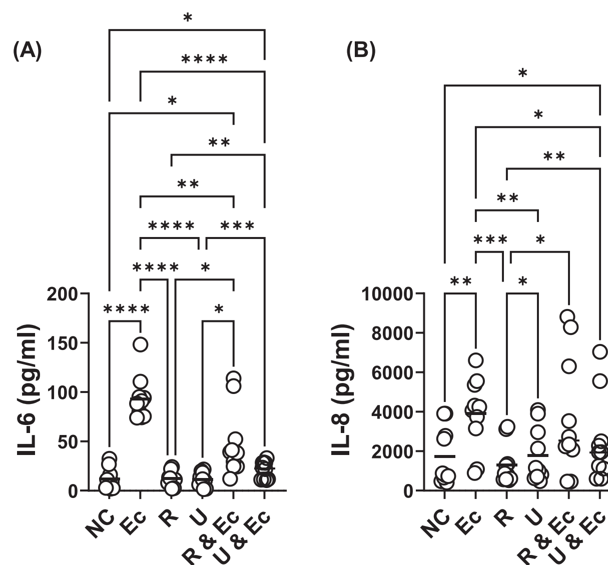

FIGURE 2 | Cytokine secretion after pectin exposure. HDIM was left untreated (NC) or exposed to *E. coli* strain HS (Ec) in the presence or absence of 0.3 mg/mL of ripe or unripe pectin (R & Ec, U & Ec). Cells alone (NC) or in the presence of pectin only (R, U) were used as controls. After 5 h, supernatants were collected and used to measure the secretion of IL-6 (A) and IL-8 (B) cytokines. Data represent five independent experiments with two replicates each; horizontal lines represent the mean values of pooled data. Paired two-tailed mixed-effects models were used to compare the multiple groups. p values < 0.05 were considered statistically significant. The levels of significance are: *, 0.01–0.05; **, 0.001–0.01; ***, 0.0001–0.001; **** < 0.0001.

of tight junction genes, such as claudins [37–39]. To explore the impact of pectins on the regulation of genes important for epithelial barrier maintenance, we used RT-PCR to analyze the expression of five tight junction genes: *CLDN1* (Claudin 1), *CLDN3* (Claudin 3), *CLDN4* (Claudin 4), *CTNNA1* (Catenin 1), and *CTNNA3* (Catenin 3). Our results revealed significant downregulation of all evaluated tight junction genes in cultures exposed to *E. coli* and treated with either ripe or unripe pectin, compared with untreated cultures (n = 3 independent experiments with 2–3 replicates each) (Figure 3).

Next, we performed unsupervised principal component analysis (PCA) to reduce data dimensionality and explore associations between the expressions of tight junction genes. Gene expression data from three independent experiments were combined to generate the PCA matrix. The analysis revealed that the first principal component (PC1, 72.65%) and the second principal component (PC2, 22.05%) together accounted for 94.7% of the total variance (Figure 4A). This dimensionality reduction clearly distinguished the groups based on the presence or absence of microbiota (*E. coli*) (Figure 4B). Next, we utilized a loading plot to investigate the relationships between individual genes further. As shown in Figure 4C, two distinct clusters were observed: one comprising *CLDN3*, *CLDN4*, and *CTNNA1*, and another comprising *CLDN1* and *CTNNA3*. This indicates that the genes within each cluster are positively correlated. However, the vectors for *CLDN1* and *CTNNA3* cluster form an almost right angle relative to the vectors for *CLDN3*, *CLDN4*, and *CTNNA1* cluster, suggesting that the genes in these two clusters might be uncorrelated. Collectively, these results demonstrate that pectins impact the epithelial barrier by downregulating tight junction genes. This downregulation involves two clusters of positively correlated genes, each potentially functioning independently, suggesting both coordinated and distinct gene responses.

3.4 | Effects of Pectins on Epithelial Cell Antibacterial Responses

To further explore the impact of pectins on the epithelial cell barrier, we examined their regulation of genes associated with the host's innate immune response to bacteria. Using the RT^2 Profiler PCR Array kit, we profiled 84 genes involved in pathways related to Toll-Like Receptor (TLR) and NOD-Like Receptor (NLR) signaling, downstream antibacterial responses, apoptosis, and antimicrobial peptide signaling via qRT-PCR. Since both ripe and unripe pectins reduced IL-6 levels and improved cell viability in cultures exposed to *E. coli*, we hypothesized that, regardless of the ripening stage, both pectins might influence the host's innate immune response to bacteria. To test this hypothesis, we conducted these experiments using ripe pectins, which showed a weaker anti-inflammatory effect than unripe pectins under the conditions described above. Among the 84 genes studied, only tumor necrosis factor receptor-associated

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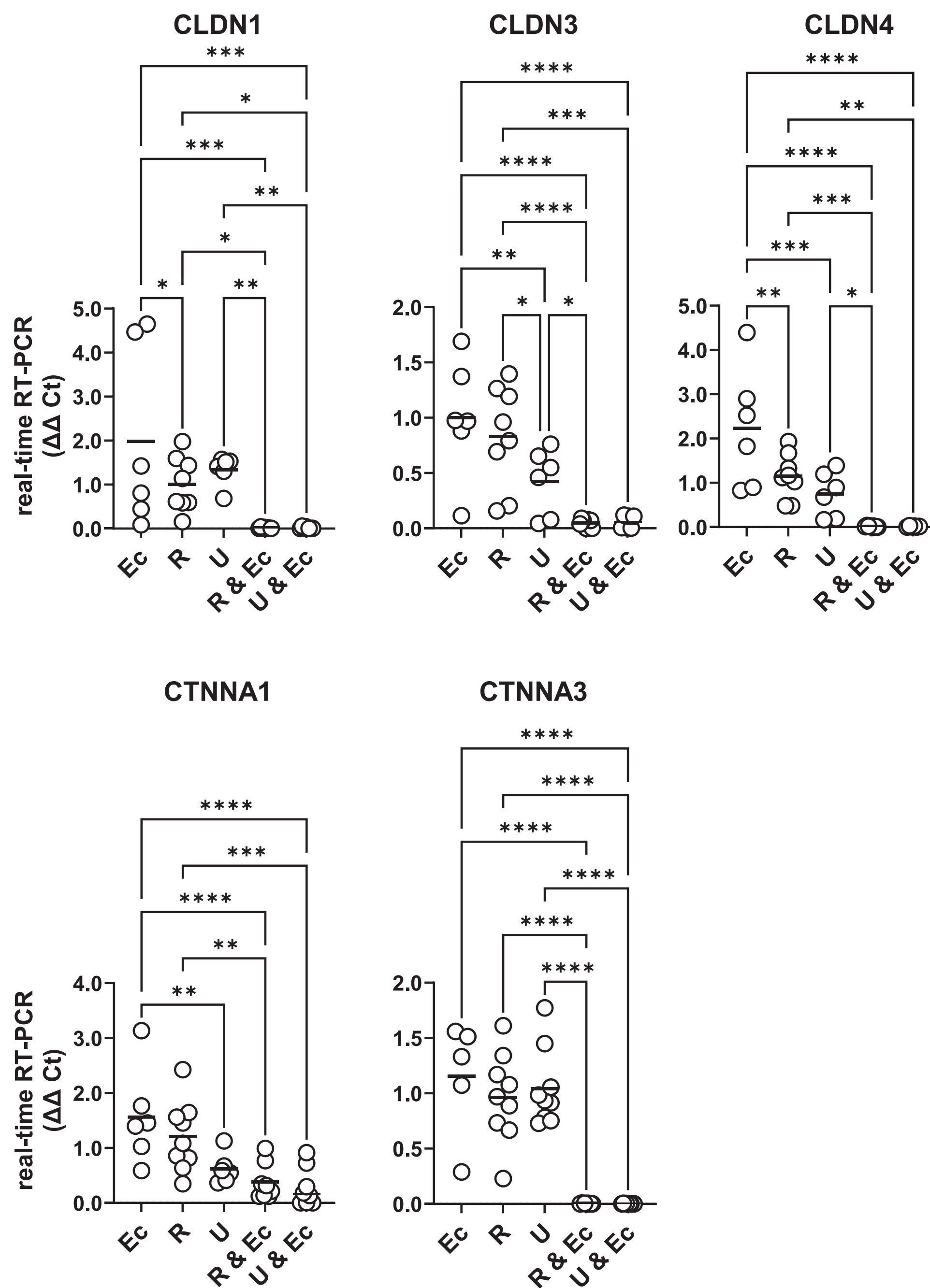

FIGURE 3 | Differential expression of tight junction genes after pectin exposure. HDIM was left untreated (NC) or exposed to *E. coli* strain HS (Ec) in the absence or presence of 0.3 mg/mL of ripe or unripe pectin (R & Ec, U & Ec). Cells alone (NC) or in the presence of pectin only (R, U) were used as controls. After 5 h, cells were harvested for the extraction of mRNA and tight junction gene expression (*CLDN1*: Claudin 1, *CLDN3*: Claudin 3, *CLDN4*: Claudin 4, *CTNNA1*: Catenin 1, and *CTNNA3*: Catenin 3) measured by real-time qPCR. Data represent three independent experiments with 2–3 replicates each; horizontal lines represent the mean values of pooled data. Paired two-tailed mixed-effects models were used to compare the multiple groups. p values < 0.05 were considered statistically significant. The levels of significance are: *, 0.01–0.05; **, 0.001–0.01; ***, 0.0001–0.001; **** < 0.0001.

factor 6 (*TRAF6*) and sodium–glucose co-transporter (*SGLT1*) exhibited significant changes greater than 2-fold in cultures exposed to *E. coli* compared to untreated controls (i.e., NC vs. Ec) ($n = 3$ independent experiments) (Figure 5). However, when cultures exposed to *E. coli* were treated with ripe pectin, these two genes showed no significant differences compared to untreated controls (i.e., NC vs. R & Ec) (Figure 5). Interestingly, when comparing cultures exposed to *E. coli* alone to those treated with ripe pectin without *E. coli* (i.e., Ec vs. R), we observed downregulation of *TRAF6*, *SUGT1*, and tumor necrosis factor receptor 1 (*TNFRS1A*), alongside upregulation of the Lipocalin-2 (*LCN2*) gene (Figure 5). Additionally, when comparing the effects of ripe pectin in cultures exposed to *E. coli* with those exposed to either *E. coli* (i.e., Ec vs. R & Ec) or ripe pectin alone (i.e., R vs. R & Ec), we observed downregulation of NLR family pyrin domain containing 3 (*NLRP3*) and upregulation of mitogen-activated protein 2 kinase 1 (*MAP2K1*), respectively (Figure 5). These findings suggest that pectins may activate

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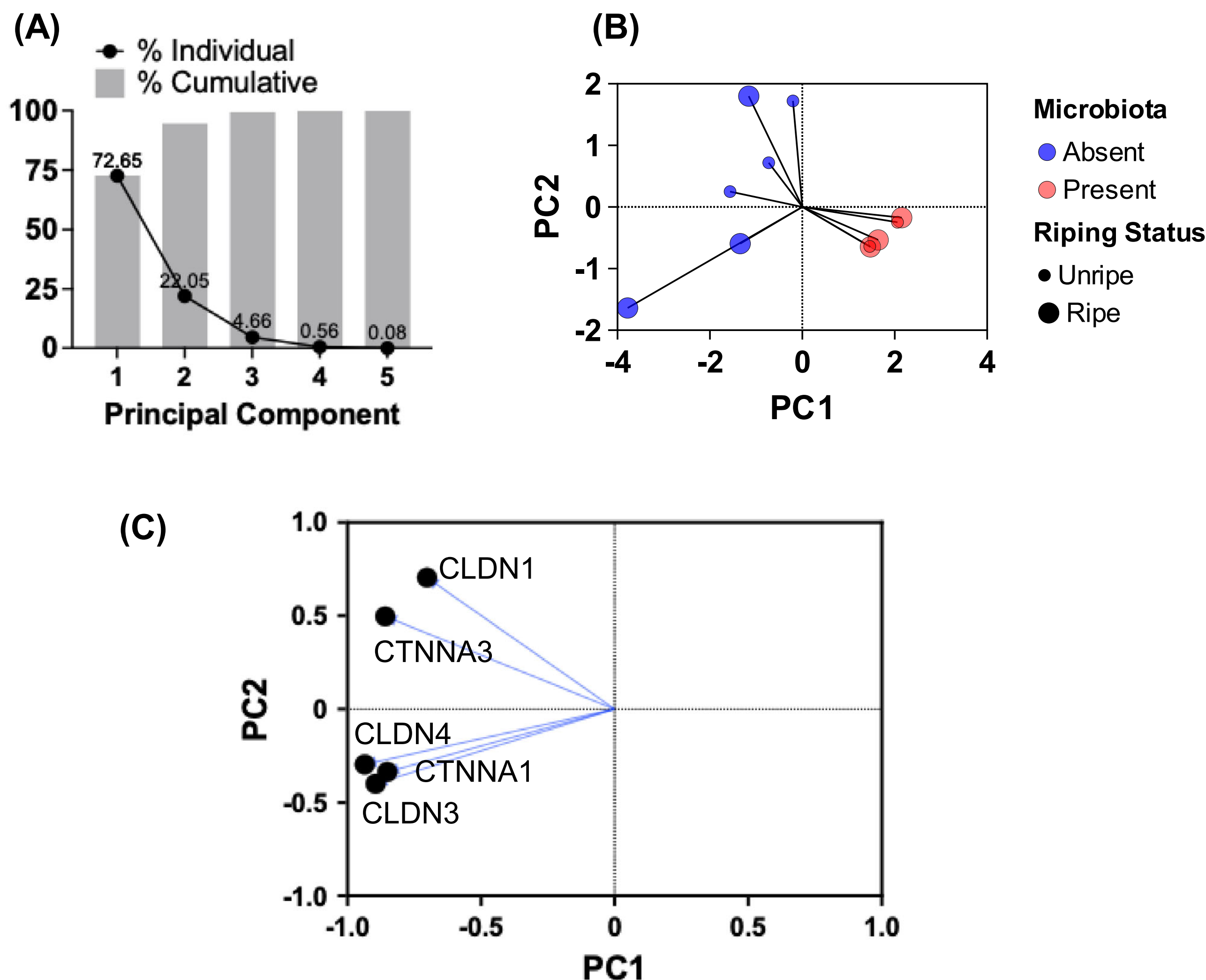

FIGURE 4 | Data integration using principal component analysis (PCA). (A) Proportion of variance. The percent variation is plotted for each component (bars) and cumulatively (line). Unit variance scaling is applied to rows; singular value decomposition (SVD) with imputation is used to calculate principal components. *X* and *Y* axes show principal component 1 (PC1) and principal component 2 (PC2) that account for 72.65% and 22.05% of the total variance, respectively. (B) PC scores. (C) PCA loadings plot showing the distributions of the analytical variables.

different pathways when in combination with the microbiota. This assumption is partially supported by the results of the tight junction gene analysis, which showed that the PCA plot clearly distinguished two clusters: one with and one without microbiota (Figure 4B).

4 | Discussion

Increasing evidence suggests that HDIM is an excellent in vitro model for studying intestinal physiology and pathology during the neonatal period [1]. It is well known that the neonatal intestinal epithelium differs from that of adults in terms of permeability, immune regulation, and microbiota interactions [40]. Additionally, HDIM simplifies the complexity of the neonatal intestine while retaining key physiological characteristics, enabling mechanistic studies that are challenging to perform in vivo due to ethical and technical constraints. Through our investigations using HDIM, we have made a significant contribution to unraveling the early molecular mechanisms underlying the interaction among papaya pectin, the neonatal gut, and the microbiota, an area that remains largely unexplored.

Our study provides evidence that, regardless of the papaya pectin ripening stage or the presence of a commensal, pectin treatment significantly reduces cell death compared to untreated controls. These findings suggest that papaya pectins may play a protective role in neonatal intestinal health by enhancing cell viability, particularly by maintaining intestinal integrity and mitigating microbial stress [41]. Interestingly, these results contrast with our previous findings, which showed that ripe papaya pectin decreased cancer cell viability and induced necroptosis [42]. A plausible explanation is that pectin's structural components, particularly RG-I and arabinogalactan-rich domains, interact with galectin-3 expressed in the neonatal intestinal epithelium in a protective rather than disruptive manner. Given that healthy neonatal intestinal cells express relatively low levels of galectin-3, they are less susceptible to pectin-induced cytotoxicity and may be better equipped to withstand inflammatory or microbial stress. In contrast, cancer cells commonly overexpress galectin-3 [42, 43],

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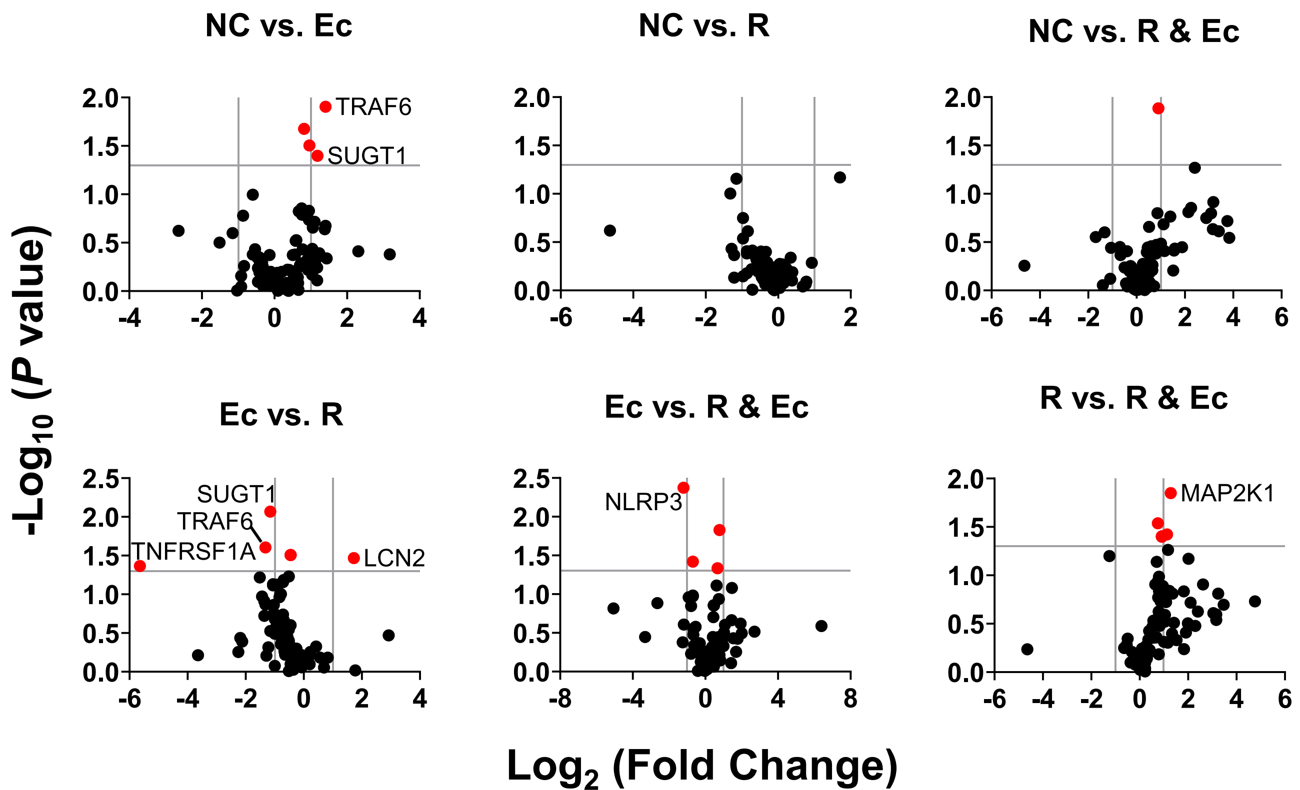

FIGURE 5 | Pectin effects on epithelial cell antibacterial responses. HDIM was left untreated (NC) or exposed to *E. coli* strain HS (Ec) in the absence or presence of 0.3 mg/mL of ripe pectin (R & Ec). Cells alone (NC) or in the presence of ripe pectin only (R) were used as controls. After 5 h, cells were harvested for extraction of mRNA and used to determine the antibacterial profiles of 84 genes by qRT-PCR. Volcano plots depicting differentially expressed gene *p* values (−log10) as a function of fold change (log2) in HDIM models. Red dots (●) indicate *p* values of < 0.05. Each dot symbolizes the log2 fold change of the experimental condition as compared to cultures with media only (controls). Data represent three independent experiments. Only genes with > 2-fold and *p* values of < 0.05 were annotated.

rendering them more vulnerable to pectin binding through these same domains. This interaction can disrupt key processes such as cell adhesion, proliferation, and survival, while leaving healthy cells largely unaffected [44].

In addition to promoting cell viability, both ripe and unripe papaya pectins significantly reduced IL-6 production in the presence of commensal *E. coli*. This is consistent with previous studies demonstrating that dietary pectins attenuate IL-6 secretion in vivo, including in a mouse model of experimental colitis [45, 46]. Interestingly, a decrease in IL-8 levels was observed only in cultures exposed to *E. coli* and treated with unripe pectin, supporting the hypothesis that the profile of anti-inflammatory responses induced by papaya pectins is influenced by ripening stage. As the fruit matures, notable changes occur in pectin composition. Papaya ripening is characterized by rapid pulp softening, primarily driven by pectin-degrading enzymes. During this process, the activity of polymethyl esterases (PMEs) and pectate lyases (PLs) decreases, whereas polygalacturonase (PG) activity increases significantly, leading to extensive depolymerization and solubilization of pectin molecules [47]. These changes result in smaller pectin fragments with altered molecular weights, which may influence their biological activity and interactions with host cells [48]. Furthermore, reduced esterification in pectins can enhance their binding affinity for pattern recognition receptors, such as Toll-like receptors (TLRs), thereby modulating anti-inflammatory responses at the epithelial level [49].

Since IL-6 can influence intestinal permeability by upregulating genes associated with epithelial barrier integrity, such as claudins [37–39], we next examined the effect of papaya pectin on the expression of tight junction genes, including *CLDN1*, *CLDN3*, *CLDN4*, *CTNNA1*, and *CTNNA3*. Surprisingly, both ripe and unripe papaya pectins influenced the epithelial barrier by downregulating all evaluated tight junction genes, which encode proteins crucial for maintaining barrier integrity and regulating paracellular permeability. However, the downregulation of tight junction genes was not associated with loss of epithelial viability, which remained equal to or higher than untreated controls, suggesting that this response reflects regulatory adaptation rather than barrier damage. This assumption is supported by previous studies showing that increased IL-6 levels enhance *CLDN2* expression and intestinal permeability. However, IL-6 knockdown via JNK siRNA transfection reduced *CLDN2* expression and further strengthened the intestinal barrier beyond that of untreated controls [37]. In addition, Koval's group found that increased *CLDN3* expression was associated with decreased alveolar trans-epithelial electrical resistance (TEER) and increased paracellular permeability. However, increased expression of *CLDN4* was sufficient to enhance alveolar epithelial TEER without affecting paracellular permeability [50]. Nonetheless, we acknowledge that persistent or excessive downregulation of tight junction genes may be detrimental, potentially compromising epithelial barrier integrity, weakening host defense mechanisms, and impairing immune maturation, particularly

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in the developing neonatal gut. Tight junction proteins such as claudins and catenins play a critical role in maintaining selective permeability and coordinating immune-epithelial signaling [51]. Disruption of these pathways has been associated with increased susceptibility to infection, chronic inflammation, and impaired immune development, especially during early life when the gut barrier and immune system are still maturing [52].

Our dimensionality reduction analysis further revealed two distinct clusters of tight junction genes, suggesting that pectin modulates barrier regulation through both coordinated and independent gene networks: *CLDN3*, *CLDN4*, and *CTNNA1* likely reflecting sealing functions, and *CLDN1* with *CTNNA3* reflecting adhesion-related regulation. While *CLDN1* is associated with tissue homeostasis, epithelial cell polarity, and cell migration [52, 53], *CLDN3* and *CLDN4* are typically co-expressed in normal epithelial cells. Their downregulation has been linked to reduced E-cadherin expression, and both function as "sealing" claudins that reinforce epithelial barrier integrity [54]. *CTNNA1* connects adherens junctions to actin filaments, supporting mechanical function, whereas *CTNNA3* has been implicated in specific tissue signaling and dynamic modulation [55, 56]. These findings underscore both the complexity with which pectins affect epithelial cell function and their potential to modulate the epithelial barrier in a context-specific manner.

Our results also suggest that pectins affect innate immune response genes. Among the 84 genes analyzed, *TRAF6* and *SGLT1* were significantly upregulated in cultures exposed to *E. coli* compared with untreated controls. However, in the presence of ripe pectin, these genes returned to baseline levels. Additionally, ripe pectin downregulated *TRAF6*, *SUGT1*, and *TNFRS1A* while upregulating *LCN2* in cultures without *E. coli*. These changes highlight pectin's ability to modulate both inflammation and antibacterial defense mechanisms, as *LCN2* plays a crucial role in limiting bacterial growth by sequestering iron. To better understand how pectins shape epithelial immunity during microbial challenge, we examined downstream signaling pathways underlying these initial immune shifts. Interestingly, when comparing cultures exposed to *E. coli* with or without ripe pectin, we observed downregulation of *NLRP3* and upregulation of *MAP2K1*. These genes are key players in inflammasome activation and mitogen-activated protein kinase signaling, respectively. Inflammasomes are innate immune receptors that organize pro-inflammatory responses upon activation by stress signals from pattern-recognition receptors (PRRs) [58]. The modulation of these pathways suggests that pectins may engage distinct signaling cascades when combined with microbiota, further supporting their role as dynamic regulators of host–microbe interactions. These findings are partially corroborated by the PCA of tight junction gene expression, which distinguished clusters based on the presence or absence of the commensal. This indicates that pectins not only act directly on epithelial cells but also modulate responses during microbial interactions [1, 41, 53].

It is important to highlight some limitations of the present study. First, our study lacks a more complex microbiota representative of both healthy and diseased neonates, such as those affected by NEC, which would help confirm and extend our findings. Although *E. coli* represents an early colonizer, it is not fully representative of the microbiota in full-term infants. At birth, full-term infants typically have a highly individualized gut microbiota, initially dominated by facultative anaerobes such as *E. coli* [28–30]. These pioneer microbes reduce oxygen levels, creating an anaerobic environment that supports the growth of obligate anaerobes such as *Bifidobacterium*, *Clostridium*, and *Bacteroides* [54]. Within a few weeks, strict anaerobes, especially *Bifidobacterium* species, become predominant [55]. Second, our system consists solely of epithelial cells. We and others have shown the crucial role of cross-talk between epithelial and immune cells in controlling chemokine production [25, 32, 56, 57]. Epithelial cells may indirectly modulate innate-like lymphocyte H3K4me3 epigenetic marks by regulating chemokine IL-18 secretion through the Histone Deacetylase 6 gene [25]. The IL-18 secretion by epithelial cells plays a pivotal role in inflammation as key mediators of macrophage recruitment and polarization (M1, pro-inflammatory; M2, anti-inflammatory), processes that are critical in both physiological and pathological inflammatory responses [58, 59]. Therefore, some effects of pectin may have been overlooked due to the absence of immune cells in the current model. Third, as with any in vitro system, a key limitation is the inability to evaluate the systemic effects of pectins. In vivo validation is essential to fully elucidate the mechanisms by which pectins operate within the complex physiological context of inflammation. Fourth, the number of neonatal intestinal tissue donors was small, limiting generalizability and reflecting only inter-individual differences, not population-level effects. Nevertheless, the consistency of responses across donors supports the conclusion that the observed effects are not donor-specific, although larger studies will be necessary to confirm this finding. Fifth, the relatively short incubation period (5 h). This timeframe was chosen to capture early host responses while minimizing confounding effects from bacterial overgrowth, which can occur in in vitro longer-term exposures with live bacteria. While 5 h has been shown to induce robust cellular activation in related to in vitro human models [23, 27, 31, 32, 34, 60], we cannot rule out the possibility that longer exposures might reveal delayed inflammatory or other barrier-related effects. Indeed, the limited number of antibacterial genes affected may reflect the relatively short exposure window and the specific pectin dose used, as both factors can significantly influence the timing and magnitude of antimicrobial responses. Finally, while IL-6 and IL-8 provided valuable insights into the epithelial inflammatory response, pectin's immunomodulatory effects are likely to involve broader and more complex signaling networks. Cytokines such as TNF-α, IL-1β, IL-10, and others may exhibit synergistic or antagonistic interactions that are not captured by a limited cytokine panel [61]. Future studies should thus employ more comprehensive profiling approaches, such as multiplex ELISA or transcriptomic analysis, to better elucidate the breadth and interplay of inflammatory mediators modulated by pectin treatment.

In summary, this study demonstrates that papaya pectins modulate intestinal epithelial barrier function in a stage- and microbial-context-dependent manner. These effects likely involve modulation of epithelial signaling pathways that govern barrier integrity and inflammatory responses. Nonetheless, these findings are based on an in vitro model of neonatal intestinal mucosa. They should be interpreted with caution, especially regarding their modulation of epithelial barrier genes and their translational relevance. Future research should employ in vivo models to investigate dose–responsiveness and host–microbe–

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pectin interactions in promoting intestinal health during early life.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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