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ORIGINAL ARTICLE

Mechanisms of gut epithelial barrier impairment caused by food emulsifiers polysorbate 20 and polysorbate 80

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Abstract

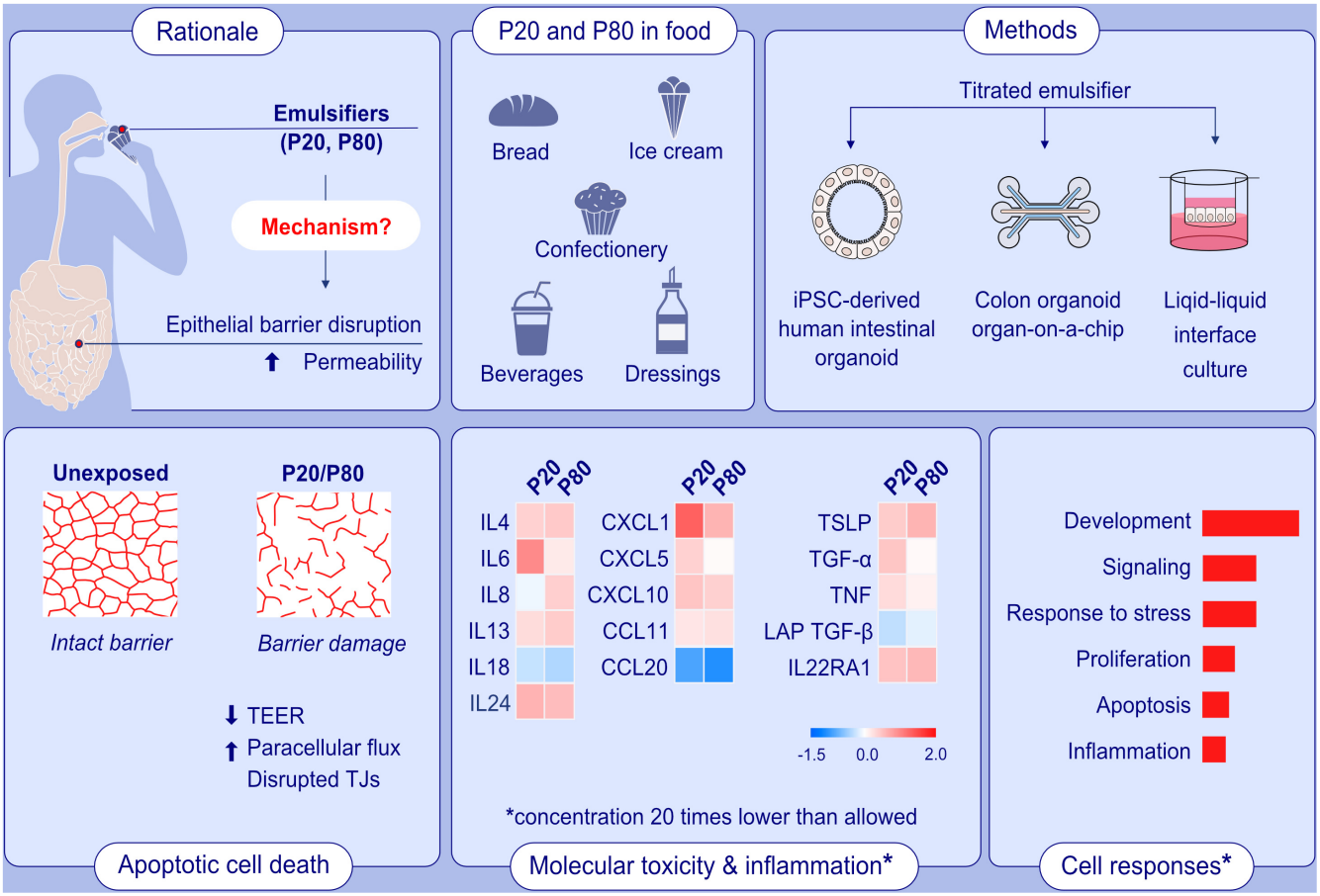
Background: The rising prevalence of many chronic diseases related to gut barrier dysfunction coincides with the increased global usage of dietary emulsifiers in recent decades. We therefore investigated the effect of the frequently used food emulsifiers on cytotoxicity, barrier function, transcriptome alterations, and protein expression in gastrointestinal epithelial cells.

Methods: Human intestinal organoids originating from induced pluripotent stem cells, colon organoid organ-on-a-chip, and liquid–liquid interface cells were cultured in the presence of two common emulsifiers: polysorbate 20 (P20) and polysorbate 80 (P80). The cytotoxicity, transepithelial electrical resistance (TEER), and paracellular-flux were measured. Immunofluorescence staining of epithelial tight-junctions (TJ), RNA-seq transcriptome, and targeted proteomics were performed.

Results: Cells showed lysis in response to P20 and P80 exposure starting at a 0.1% (v/v) concentration across all models. Epithelial barrier disruption correlated with decreased TEER, increased paracellular-flux and irregular TJ immunostaining. RNA-seq and targeted proteomics analyses demonstrated upregulation of cell development, signaling, proliferation, apoptosis, inflammatory response, and response to stress at 0.05%, a concentration lower than direct cell toxicity. A proinflammatory response was characterized by the secretion of several cytokines and chemokines, interaction with their receptors, and PI3K-Akt and MAPK signaling pathways. CXCL5, CXCL10, and VEGFA were upregulated in response to P20 and CXCL1, CXCL8 (IL-8), CXCL10, LIF in response to P80.

Conclusions: The present study provides direct evidence on the detrimental effects of food emulsifiers P20 and P80 on intestinal epithelial integrity. The underlying mechanism of epithelial barrier disruption was cell death at concentrations between 1% and 0.1%. Even at concentrations lower than 0.1%, these polysorbates induced a proinflammatory response suggesting a detrimental effect on gastrointestinal health.

KEYWORDS
epithelial barrier, food emulsifiers, polysorbate-20, polysorbate-80, RNA-seq, targeted proteomics



GRAPHICAL ABSTRACT

This study provides new insights into the underlying mechanisms of intestinal epithelial barrier defects in response to commonly used food emulsifiers P20 and P80. We demonstrated that P20 and P80 directly impair barrier integrity of gut epithelial cells and cause molecular toxicity and proinflammation at doses 20 times lower than those currently authorized for use. At RNA transcription and protein levels, development, cell signaling, proliferation, apoptosis, inflammation and response to stress were altered. Abbreviations: CCL, C-C motif ligand; CXCL, C-X-C motif ligand; IL, interleukin; IL22RA1, interleukin 22 receptor subunit alpha 1; iPSC, induced pluripotent stem cell; LAP, latency-associated peptide; P20/P80, polysorbate 20/polysorbate 80; TEER, transepithelial electrical resistance; TGF-β, transforming growth factor beta; TNF, tumor necrosis factor; TJs, tight junctions.

1 | INTRODUCTION

The "epithelial barrier theory" proposes that hazardous substances introduced into our bodies through a combination of dietary and lifestyle habits stress the epithelial lining and thereby contributes to an increased barrier permeability, microbial dysbiosis, translocation of bacteria to inter- and subepithelial areas, tissue microinflammation, and a proinflammatory immune response.[1–3] Of particular, interest are "emulsifiers," which are among the most extensively used food additives[4] and are used to stabilize functional components and flavorings, subsequently improving shelf life.[5] Since the mid-20th century, there has been a steep rise in the consumption of processed foods containing emulsifiers that coincides with an increase in the prevalence of various chronic inflammatory disorders[6] such as Crohn's disease,[7,8] ulcerative colitis,[9] diabetes,[10] obesity, and metabolic syndrome.[10,11]

Previous in vitro studies reported that non-ionic surfactant food emulsifiers, including polysorbates, (1) demonstrate concentration-dependent effects on intestinal cell permeability and cell viability,[12] (2) induce bacterial translocation across M-cells,[13] (3) cause structural and functional damages to mitochondria in enterocytes,[14] and (4) enhance the absorption of allergens in a size-dependent manner.[15] Emulsifiers can directly affect the microbiota by increasing its proinflammatory potential and decreasing its capacity for fiber fermentation.[16–18] The relationship between emulsifiers and gut microbiota has promoted colitis in mice, disturbed intestinal permeability and mucus structure, and caused microbial dysbiosis.[19–24] Recently, a double-blind controlled human feeding study reported an increase in the prevalence of chronic inflammatory diseases by affecting the gut microbiota and metabolome.[25]

Despite these previous in vitro and in vivo studies, however, the direct impact of emulsifiers at varying concentrations on host gene expression remains opaque. In this study, we systematically tested the impact of two well-known food emulsifiers—polysorbate 20 (P20, E432) and polysorbate 80 (P80, E433)—on gut barrier integrity. P20 and P80 are added into ice cream, beverages, dressings, bread, and confectionery at concentrations of up to 1%. Their usage at these

levels is generally regarded as safe for human consumption by regulatory bodies. Their purity parameters, toxicological data, methods of analysis, and examples of applications have been reported in detail.[26,27]

We used human intestinal organoids, colon organoid organ-on-a-chips, liquid–liquid interface cultures, and transcriptome and targeted proteomics analyses of the intestinal epithelial cells to observe the impact of variable emulsifier concentrations on gut physiology. We observed that P20 and P80 dramatically disrupt the gut barrier even at well below the approved concentrations. Overall, this effort provided insights into the doses and molecular mechanisms by which P20 and P80 open epithelial barriers, induce apoptotic cell death, and initiate inflammatory cascade on epithelial cells, the epithelitis.

2 | MATERIALS AND METHODS

Detailed descriptions of methods and reagents are available in the Methods S1.

2.1 | Cell cultures

Caco-2 cells were obtained from the American Type Culture Collection and used between passages 20–45 in all experiments. 3-Lane 64 OrganoPlates were used to culture the gut-on-a-chip models. Human induced pluripotent stem cell (iPSC)-derived intestinal organoids were obtained from DefiniGEN and were used between passages 4–6. For studying the effects of emulsifiers, organoids mixed with Cultrex UltiMatrix (R&D Systems) were seeded onto a glass-bottom 96-well plate (Thermo Fisher) and allowed to grow for 6–7 days before the indicated treatments were performed. For details, please see Methods S1.

2.2 | RNA-sequencing and data analysis

Total RNAs were isolated using an RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's protocol. Total RNA quantity and quality were determined using 2200 Tape Station Automated Electrophoresis System (Agilent Technologies). Samples with an RNA integrity number of greater than 9.8 were chosen for sequencing. RNA-seq was performed with the TruSeq Stranded mRNA Sample Prep Kit (Illumina) on the Illumina NovaSeq 6000. All sequencing datasets are publicly available at the NCBI Gene Expression Omnibus under accession number (GSE227220).

Low-quality tails and adapters were trimmed from reads before read alignment. The obtained dataset was aligned to the Ensembl genome reference, release 38 (GRCh38.p13) by STAR aligner (version 2.7.9a). Gene expression values were quantified by feature counts implemented in the Rsubread software (Bioconductor). Differentially expressed genes were assessed by DESeq2 (version 1.32.0). Genes with a p value of less than .01 and $\log_2$ fold change of greater than 1 or less than −1 were included in this study. Gene Ontology (GO) categories were performed using the Bioconductor package GOSeq with the Wallenius approximation.

2.3 | Protein quantification and data analysis

Supernatants from the Caco-2 monolayer cultures were analyzed using targeted proteomics via the proximity extension assay (Olink), according to the manufacturer's instructions. Panels included analytes associated with immune response and inflammation. Each panel consisted of 92 separate analytes.

The Olink-generated data were preprocessed and quality-controlled using the platform-specific "Olink NPX manager" software, which corrects background, $\log_2$ transforms and normalizes all samples to an arbitrary normalized protein expression (NPX) scale. Additional Olink data were analyzed using R (version 4.2.0). Differential expression analysis of proteins was achieved using the limma package (version 3.52.2). Benjamini–Hochberg's false discovery rate corrected q-values were calculated to correct for multiple testing in all parts of the differential expression analysis. Proteins with an adjusted p values <.05 and an absolute estimate >2 were considered significantly differential biomarkers. Functional analysis of the proteins identified was conducted using GO annotation, and proteins were categorized according to their biological processes.

In addition, the weighted co-expression network analysis (WGCNA) R package was used to create a weighted protein correlation network. WGCNA adjacency function was used to produce a weighted network adjacency matrix with parameters "type = signed" and "power = 15." To achieve approximate scale-free topology, soft-thresholding power was chosen as the lowest power. Then, a topological overlap matrix of dissimilarity was defined using the TOMdist function. Clusters of interconnected proteins were found using hierarchical clustering and the cutreeDynamic function with parameters: method = "hybrid", deepSplit = 2, minClusterSize = 10. The association of these clusters with variables was assessed. We defined hub genes as those with a high module membership (|cor.weighted| > 0.9) as hub genes.

2.4 | Statistical analysis

Differences between paired groups were evaluated by using the Wilcoxon signed-rank test. All statistical analyses and associated figures were generated with GraphPad Prism, version 9.0 (GraphPad) or R package. Differences were considered significant with p values <.05.

3 | RESULTS

3.1 | P20 and P80 show cytotoxicity and impair epithelial barrier function and integrity on gut epithelial cells

To investigate the potential impact of commonly used emulsifiers, we first determined P20- and P80-mediated cytotoxicity of Caco-2 cells after 24 h exposure. Phase-contrast images showed detachment and breakdown of the cytosolic membrane of cells at concentrations of ≥0.25% (v/v) for P20 and P80 compared to unexposed

cells (Figure 1A). Using the conventional lactate dehydrogenase (LDH) assay, dose-dependent cytotoxicity was found in monolayer-cultured Caco-2 cells. Starting at a concentration of 0.1%, cell lysis was observed in response to P20 and P80 exposure in a dose-dependent manner. P20 and P80 at low concentrations (0.05%) did not exert cytotoxic effects (Figure 1B). Therefore, the concentration of P20 and P80 applied in the RNA-seq and targeted proteomics experiments were used at 0.05% to make sure that the cell survival was not notably inhibited compared to the control group.

We used a liquid–liquid culture model on a Transwell system to investigate the potential barrier disruption induced by P20 and P80 on Caco-2 cells. The transepithelial electrical resistance (TEER) and paracellular flux of differentiated Caco-2 cells were measured when treated with in a concentration-dependent manner. A sharp decreases in TEER and an increase in paracellular flux were observed at concentrations of ≥0.5% for P20 and ≥1% for P80 (Figure 1C,D). In addition, P20 induced a decrease in TEER and a significant increase in paracellular flux at concentrations of 0.1% and 0.25%. Similarly, P80 also reduced TEER and increased paracellular flux at concentrations of 0.25% and 0.5%. There was no significant difference in average TEER and paracellular flux values observed upon treatment with P20 at 0.05% and P80 at 0.1% and 0.05% concentrations (Figure 1C,D). Overall analyses of the data showed the toxic threshold of P20 is lower than P80.

We further assessed the immunofluorescent staining to study the damage to tight junction (TJ) proteins claudin-1 and occludin by P20 and P80 at the same concentrations. After 3-day exposure to the emulsifiers, irregular and heterogenous staining were observed at the concentrations of 0.25 and 0.1% by P20, and 0.5 and 0.25% by P80. Complete cell lysis was observed at the concentrations of 1 and 0.5% for P20 and 1% for P80 (Figure 1E). These results demonstrate that both food emulsifiers impaired barrier integrity in gut epithelial cells.

Organ-on-a-chip models were then used to support these findings. This is 3D approach that offers different perfusable microchannels in which colon organoids or Caco-2 cells can be cultured in an organ-like orientation using the support of an extracellular matrix gel.[28] Because the polarized apical side of enterocytes face toward the inside of the organoids and the basolateral side is in contact with the medium, P20 and P80 were added to the basolateral side of the organoid cultures. This was because of the nature of the growth of organoids having the apical side of the cells inside of the organoid. To eliminate this disadvantage of organoids and to investigate the effects of P20 and P80 on basolateral sides of the intestinal organoids, experiments were continued to be performed in colon organoid organ-on-a-chip model. Gut barrier studies on colon organoid organ-on-a-chip model showed that TEER values sharply decreased and paracellular flux increased at concentrations of ≥0.25% for P20 and ≥0.5% for P80 (Figure 2A,B). Morphological changes in parallel organo-chips indicating severe cellular damage were observed with exposure at concentrations above 0.25% for P20 and P80, and irregular and heterogeneous staining of claudin-1 and occludin at concentrations 0.1% for P20 and P80 were visible (Figure 2C). Same findings of TEER results were observed in Caco-2 organ-on-a-chips. Irregular and heterogeneous staining of occludin at 0.1% for P20 and 0.25% for P80 were also shown (Figure S1). These findings using organo-chips support epithelial barrier damage by P20 and P80 as previously observed at the same concentrations TEER measurements and immunofluorescent staining.

Gut organoids have advantages over liquid–liquid interface cultures as they mimic the organ structure and the complete cellular differentiation is closer to the natural state of the organs. Accordingly, we used iPSC-derived human intestinal organoids to investigate the toxic effects of P20 and P80. Since the natural growth of organoids occurs on the apical side of the cells inside the organoids, we treated the basolateral side of the organoids with P20 and P80 at the same concentrations. The paracellular permeability of intestinal organoids was evaluated using 4 kDa TRITC-labeled and 150 kDa FITC-labeled dextrans. Our results showed that the TJs of intestinal organoids were permeable to 4 kDa dextran (red) but not permeable to 150 kDa dextran (green) compared to controls. However, the organoids became permeable to 150 kDa dextran in response to both emulsifiers starting at a 0.05% concentration (Figure 3). In accordance with the aforementioned results, P20 and P80 induced organoid damage at 1% and 0.5% concentrations (Figure 3). According to LDH assay, cell lysis was observed starting at a concentration of 0.1% in response to P20 and P80. P20 and P80 at 0.05% concentration did not exert cytotoxic effects (Figure S2). These findings demonstrate that P20 and P80 induced cell death and impaired the epithelial barrier in iPSC-derived human intestinal organoids in a dose-dependent manner.

3.2 | P20 and P80 alter the transcriptome in gut epithelial cells

RNA-seq was performed to analyze the differential gene expression on monolayer-cultured Caco-2 cells after exposure to P20 and P80 for 24 h. In agreement with the cytotoxicity, TEER, paracellular flux, and confocal microscopy findings, the Caco-2 cells were exposed to 0.05% concentration, a non-cytotoxic dose. RNA-seq results showed differential expression of 1665 genes in response to P20 and 651 genes in response to P80 compared to unexposed controls (Figure 4A,B). The differential expression of the genes was analyzed by GO term enrichment to identify the biological pathways affected by P20 and P80. The significantly upregulated pathways included cell differentiation, communication, proliferation, adhesion, migration, apoptosis, and response to stress in response to both P20 and P80 exposures. In addition, genes associated with response to cytokine stimulus in response to P20 and response to wounding in response to P80 were significantly upregulated (Figure 4C). Interestingly, cell development in response to both P20 and P80, and cell adhesion in response to P20 were up- and downregulated at the same time. Moreover, cytoskeleton organization and pathways implicated in the response to toxic substances were downregulated by exposure to P20. Genes involved in these pathways are listed in Table S1. Although similar pathways were involved, the number of

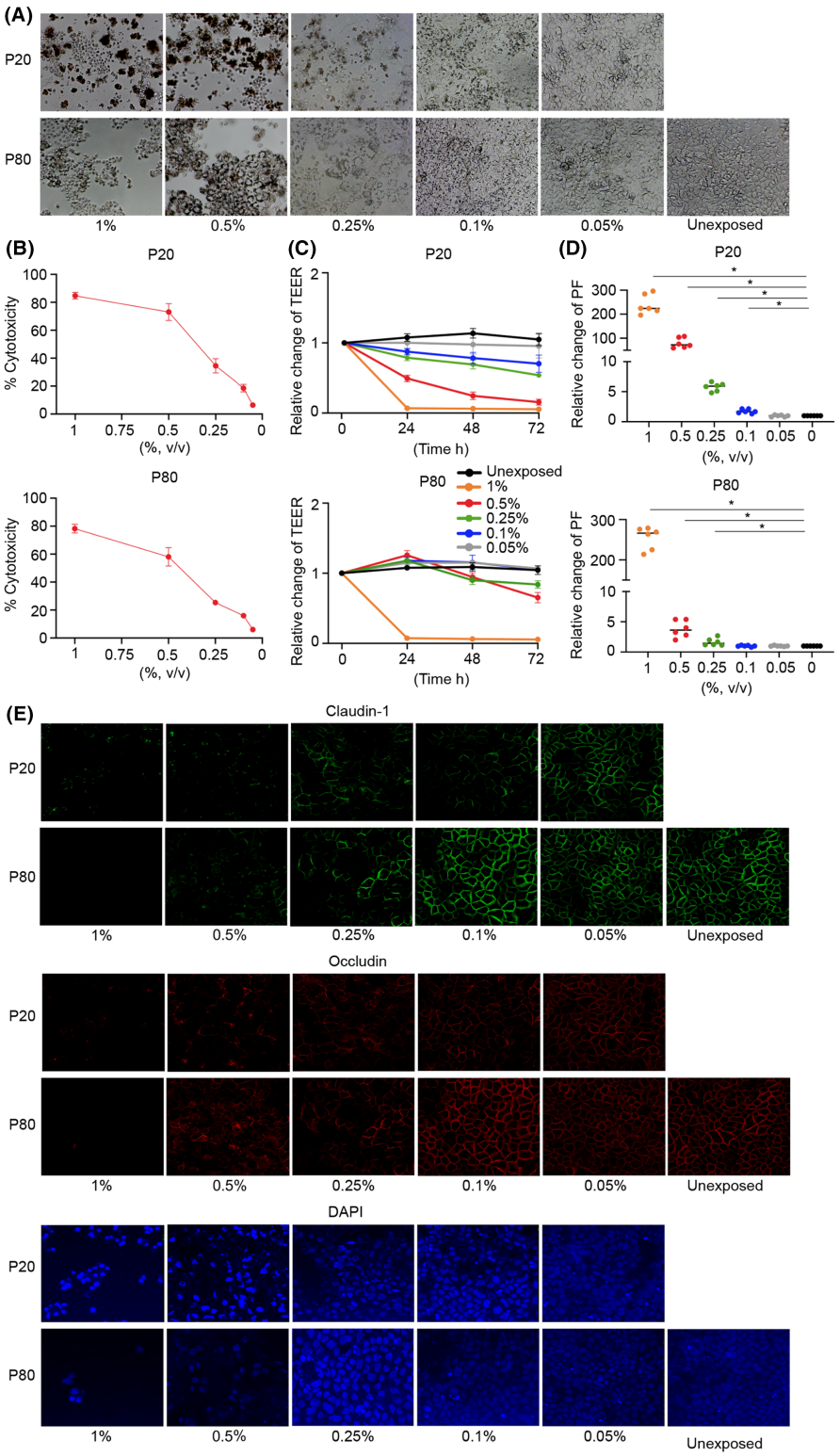


FIGURE 1 P20 and P80 showed dose-dependent cytotoxicity, decreased transepithelial electrical resistance (TEER), and increased paracellular flux (PF) and disruption of barrier integrity. (A) Representative phase-contrast images of Caco-2 monolayers after exposure to P20 and P80 for 24 h. Concentrations are labeled at the top. (B) LDH cytotoxicity of Caco-2 monolayers after exposure to P20 and P80 for 24 h at different concentrations. Data are presented as means ± SEM ($n = 3$ per group in duplicate cultures). (C) TEER was measured every 24 h for 3 days in liquid–liquid interface cultures. (D) PF was measured after exposure to P20 and P80 for 72 h. Data are presented as means ± SEM ($n = 6$ across concentrations). $^{*}p < .05$, Wilcoxon matched-pairs test. (E) Immunofluorescence staining of claudin-1 (green), occludin (red) and DAPI on liquid–liquid interface cultures after exposure to P20 and P80 at different concentrations for 72 h. A representative image of six different staining is shown.

transcripts that changed in response to P20 appeared to be almost three times higher compared to P80.

Within the 435 mRNAs that were differentially expressed by the two polysorbates, 268 were up and 167 were downregulated, and two were oppositely regulated. The differentially expressed genes involved in cell adhesion, cellular response to extracellular stimulus, regulation of lipid biosynthetic process, wound healing and epithelial cell proliferation pathways were upregulated demonstrating a common mechanism of polysorbate surfactants on cell differentiation, adhesion, proliferation, and death (Figure 4D). In contrast, there were no downregulated biological pathways found to be regulated by the two polysorbates.

In addition, this proinflammatory response was characterized by the differential expression of the genes involved in signaling

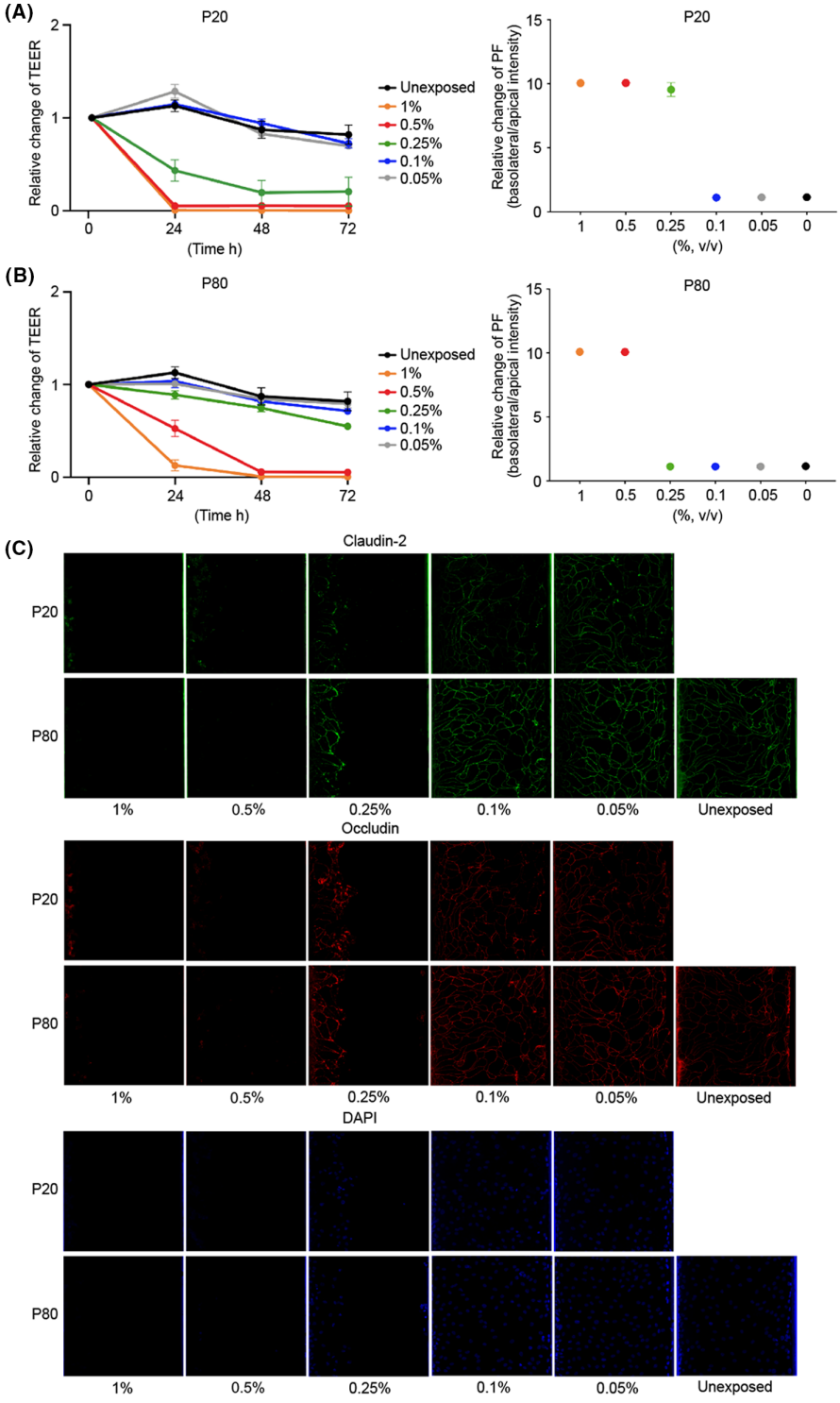


FIGURE 2 P20 and P80 showed decreased transepithelial electrical resistance (TEER) and disrupted barrier integrity in a 3D colon organoid organ-on-a-chip model. (A, B) TEER was measured every 24 h for 3 days on chips treated with P20 and P80. PF was measured after exposure to P20 and P80 for 72 h. Data are presented as means ± SEM ($n = 5$ across concentrations). (C) Immunofluorescence staining of claudin-1 (green), occludin (red) and DAPI in cells exposed to P20 and P80 at different concentrations for 72 h. A representative image of three different staining is shown.

pathways related to cytokine-cytokine interactions, chemokines, PI3K-Akt, and MAPK (Figure 4E and Figure S3). PI3K-Akt and MAPK pathways are implicated in several cellular processes including proliferation, growth and angiogenesis, cell differentiation, apoptosis, cell survival, cell motility, metabolism, response to stress and inflammation.

We performed pathway analysis using KEGG, Reactome and Wikipathways databases to investigate the impact of P20 and P80 on specific pathways. Notably, P20 exhibited a prominent association with the ferroptosis pathway. The upregulation of cysteine/glutamate transporter (SLCA11) and prion protein (PRNP), along with the downregulation of ceruloplasmin (CP), suggests a protective response against ferroptosis. Furthermore, the activation of the p38 MAPK signaling pathway in both P20 and P80-treated cells underlines its significance in the cellular stress and inflammation response. P20's effect on alanine, aspartate, and glutamate metabolism could affect energy production and cell proliferation, while changes in the Wnt pathway might influence inflammation. P80 altered lipid

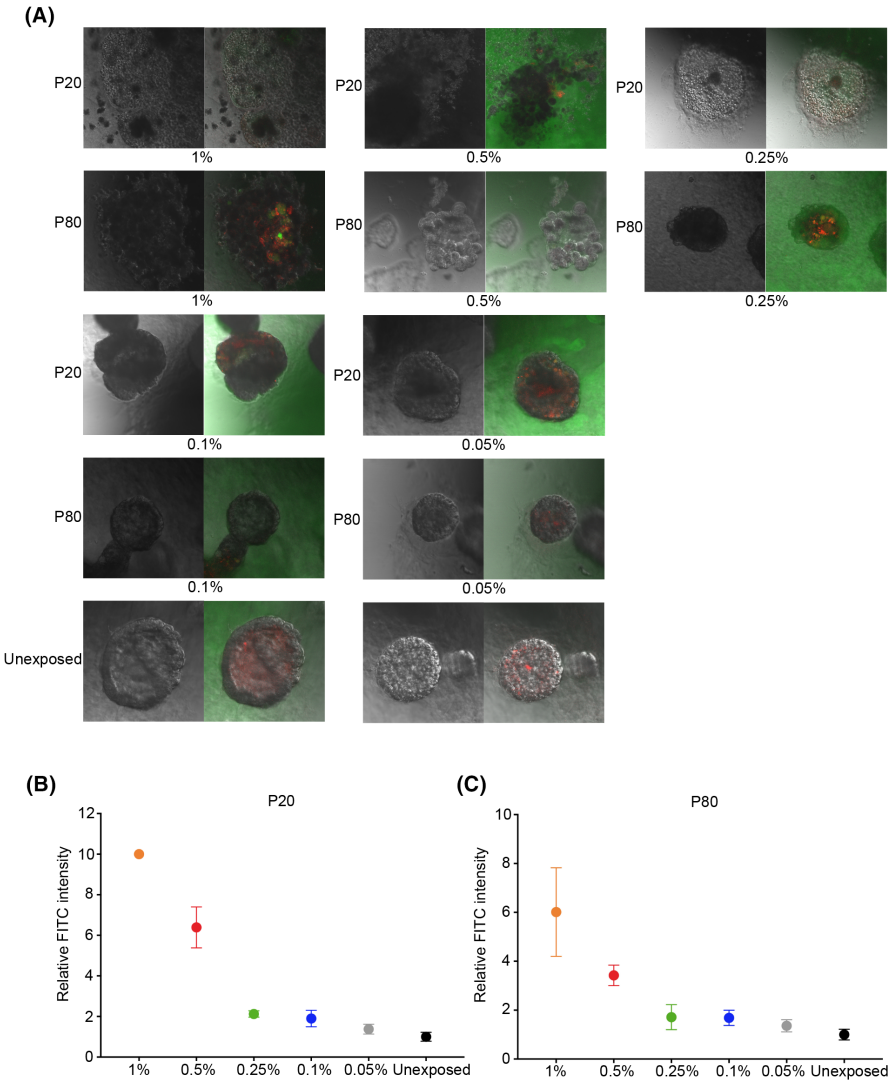


FIGURE 3 P20 and P80 disrupted the epithelial cells in iPSC-derived intestinal organoids. (A) Representative confocal microscopy images of organoid cultures treated with P20 and P80 for 24 h at different concentrations. Concentrations are labeled at the bottom of the images. 4 kDa and 150 kDa FITC-dextran passage toward inside to the lumen of organoids have been studied and merged photos have been shown on the right side of the figure. (B, C) Relative FITC intensity exposed to P20 and P80 at different concentrations.

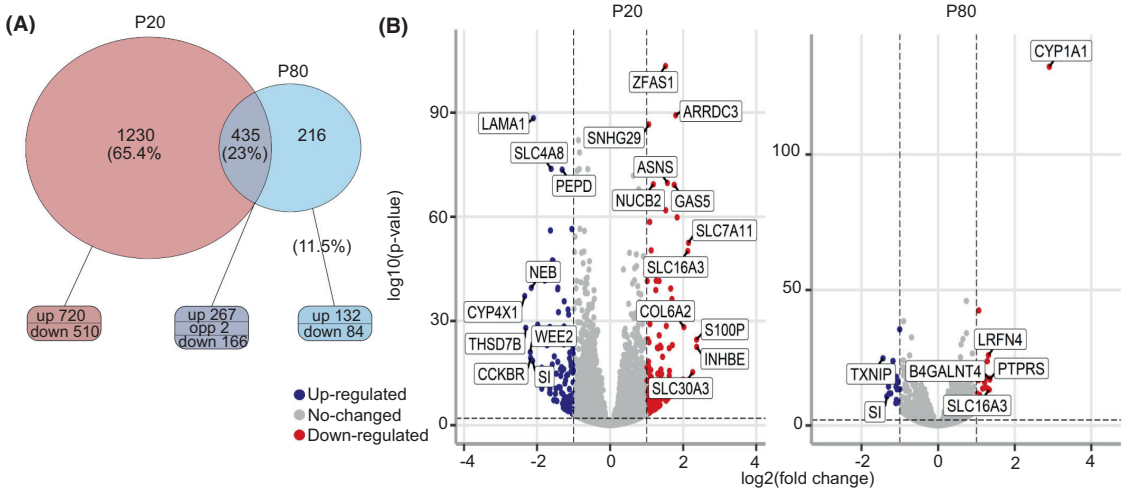
metabolism through PPAR modulation, possibly affecting lipid balance and inflammation. P80 also impacted the AHR pathway, cellular senescence, extracellular matrix proteoglycans, and diacylglycerol-derived arachidonate generation (Figure S4).

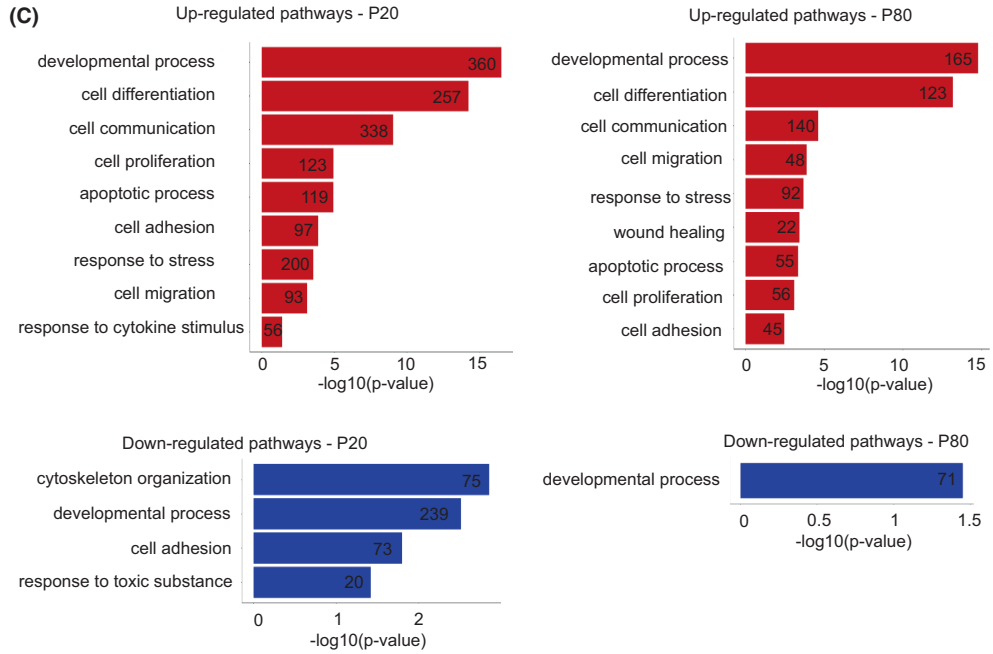
3.3 | P20 and P80 strongly affect the mRNA expression of tight junctions and adherence junctions

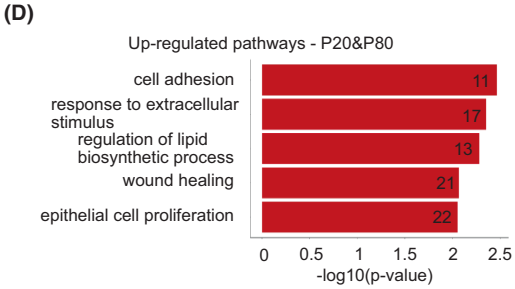
RNA-seq transcriptome analysis in response to P20 and P80 supports the disruption of the TJs and adherence junctions (AJs) as indicated by the upregulation or downregulation of many mRNAs (Figure S5). Within these molecules nine of them are significantly upregulated and 19 are downregulated. The barrier-forming TJs that were downregulated were identified as CGN, TJP1, TJAP1 in response to both emulsifiers, CLDN19 in response to P20, and OCLN and MARVELD3 in response to P80. Within the increased TJs, CLDN2 is a pore-forming protein in response to P80 and its elevated expression impairs the gut barriers. The regulated barrier- and pore-forming TJ molecules are listed in Figure S5C. The Caco-2 transcriptome analysis in response to P20 and P80 supports disruption of the AJs as indicated by upregulation of some mRNAs such as CEACAM1, ESAM, NOTCH1, VEGFA, ITGA6, PVR, PARD3, and FLOT1, and downregulation of SORBS1, ADD1, LMO7, DLG1, PLEKHA7, LAMA3, FRMD4B, MTSS1, and CDH6 at a 0.05% concentration. It is worth noting that this dose is 20 times lower than the currently authorized P20 and P80 doses (1%). These findings demonstrate a strong detrimental effect on the expression gut epithelial barrier molecules expression in response to P20 and P80 and damage to intercellular junctions as indicated by the decreased TEER and increased FITC-labeled dextran flux discussed above.

3.4 | Targeted proteomics of gut epithelial cells in response to P20 and P80

The proximity extension assay was performed with the inflammation and immune response panels (180 proteins) to analyze differentially expressed proteins and biological pathways in response to P20 and P80 at 0.05% concentration. These two panels showed a significant







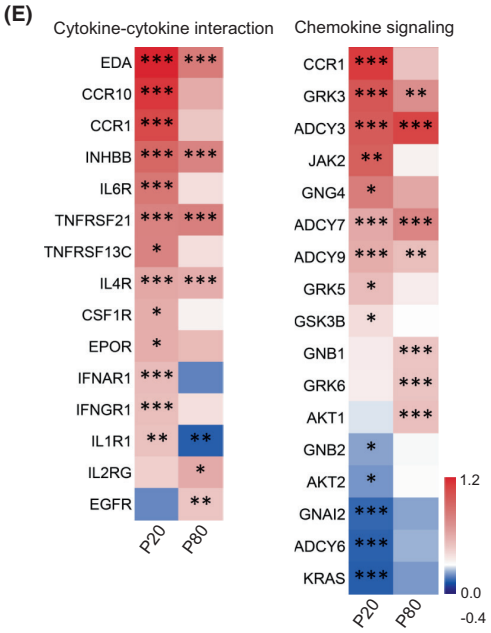


FIGURE 4 Summary of RNA-seq results in response to P20 and P80 in monolayer cultured Caco-2 cells. (A) Venn diagram showing the number of differentially expressed genes in response to P20 ($n = 5$) and P80 ($n = 5$) at 0.05% concentration after 24 h. (B) Volcano plots showing differentially expressed genes. Upregulated genes are marked in red and downregulated genes are shown in blue. The *x*-axis shows log2(fold change) in expression and the *y*-axis log10(*p* value) of differentially expressed genes. (C) Significantly upregulated and downregulated genes in response to P20 and P80 at 0.05% concentration were analyzed for pathway enrichment according to GO biological process. (D) Parallelly upregulated genes in response to P20 and P80 at 0.05% concentration were analyzed for pathway enrichment according to GO biological process. (E) Genes involved in cytokine-cytokine interaction and chemokine signaling pathways that are significantly altered in response to P20 and P80 vs unexposed (log2 ratio). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

difference in 37 proteins in response to P20, and in 34 proteins in response to P80 (Figure 5A,B). The heatmap of the differentially expressed proteins is shown in Figure S6. A strong proinflammatory response was observed with the release of several chemokines and cytokines. As observed in RNA-seq transcriptome data, response to P20 suggests a stronger proinflammatory response compared to P80. Exposure to P80 stimulated TSLP and IL-13 release that can contribute to a type 2 response, whereas P20 stimulated TNF-α, IL-6, CXCL1, and CXCL5, a more proinflammatory response. Interestingly, P20 downregulated LAP TGF-β1, MCP1, MMP1, DCBL2, MMP10, which are important for wound healing, suppression of inflammation and tissue remodeling. The expression level of PRDX5, an important protein involved in the induction and mitigation of oxidative stress, was also negatively regulated by both polysorbates.

Biological pathways related to cytokine-mediated signaling, tissue development, and response to organic substances were significantly enriched in the differentially expressed proteins in response to both P20 and P80. In addition, proteomic analysis indicated upregulation of epithelial cell apoptosis, cell proliferation, and an inflammatory response to P20 (Figure 5C). KEGG pathway analysis further confirmed the RNA-seq data demonstrating changes in cytokine and chemokine interactions, and PI3K-Akt and MAPK signaling pathways (Figure S7).

Detailed analysis of targeted proteomics data indicated that the expression patterns were indicative of a Th1-prone immune response, potential neutrophil recruitment, tissue damage and/or remodeling, and immune cell activation in response to P20. These findings may reflect an acute inflammatory response (Table S2). On the contrary, a distinct inflammatory profile was observed, potentially be linked to the cells entering a senescent state, influenced by a decrease in proteins involved in reactive oxygen species (ROS) scavenging, such as PRDX1 and PRDX5. Notably, the increase in certain molecules, including HGF and TSLP, suggests a shift toward tissue repair efforts and a Th2-based immune response, which is commonly associated with chronic inflammation (Table S3).

3.5 | Hub proteins associated with exposure to P20 and P80

Weighted co-expression network analysis was used to increase the power and reduce the multiple testing burden. This analysis was performed separately for P20 and P80, and six modules were identified in response to P20 and five modules in response to P80. The list of proteins in each module is shown in Table S4. In the P20 exposure-related proteins, the blue module was significantly associated with exposed/unexposed status. In the P80 exposure-related proteins, the blue and green modules were significantly associated with the exposed/unexposed status. To highlight putative key proteins in a data-driven manner, we identified hub proteins, defined as those that are highly interconnected in the proteomic network defined by WGCNA, including CXCL5 and CXCL10 and VEGFA in response to P20 (Figure 6A,B). The response to P80 identified CXCL1, CXCL8 (IL-8), CXCL10, and LIF as upregulated proteins, and AXIN1, CASP8, CCL20, DFFA, EIF4G1, HNMT, IL-18, MGMT, PRDX1, PRDX5, SRPK2, SULT1A1, and STAMBP as downregulated proteins in response to P80 (Figure 6A,C). The biological functions of these hub proteins are tabulated in Table S5.

4 | DISCUSSION

The extensive—and still increasing—usage of food additives in ultra-processed foods, particularly after the 2000s has been paralleled by an increase in the incidence of chronic inflammatory diseases. Previous inquiry has demonstrated that ingredients of such foods can disrupt intestinal homeostasis, thus promoting local and systemic inflammatory responses.[1–3,29] An impaired intestinal epithelial barrier function is one of the key risk factors in the pathogenesis of these diseases. The present study provides direct evidence for the detrimental effects of food emulsifiers P20 and P80 on intestinal epithelial integrity and inflammation. Using iPSC-derived human intestinal organoids, colon organoid organ-on-a-chips and liquid–liquid interface cultures we demonstrated that P20 and P80 cause epithelial barrier damage and epithelial inflammation in a concentration-dependent manner and directly impair barrier integrity of gut epithelial cells. RNA-sequencing transcriptome and multiplex proximity extension assay data revealed that the biological processes including development, cell signaling, proliferation, apoptosis, inflammatory response, and response to oxidative stress were upregulated in response to P20 and P80.

There are some studies reported on in vivo effects of P80 at 1% concentration. Chassaing et al.[19] described that P80 induced low-grade inflammation and obesity/metabolic syndrome in wild-type mice and promoted robust colitis in mice predisposed to this disorder. P80-induced metabolic syndrome was associated with microbiota encroachment, altered species composition, and increased proinflammatory potential. Furuhashi et al.[23] showed that P80 at 1%

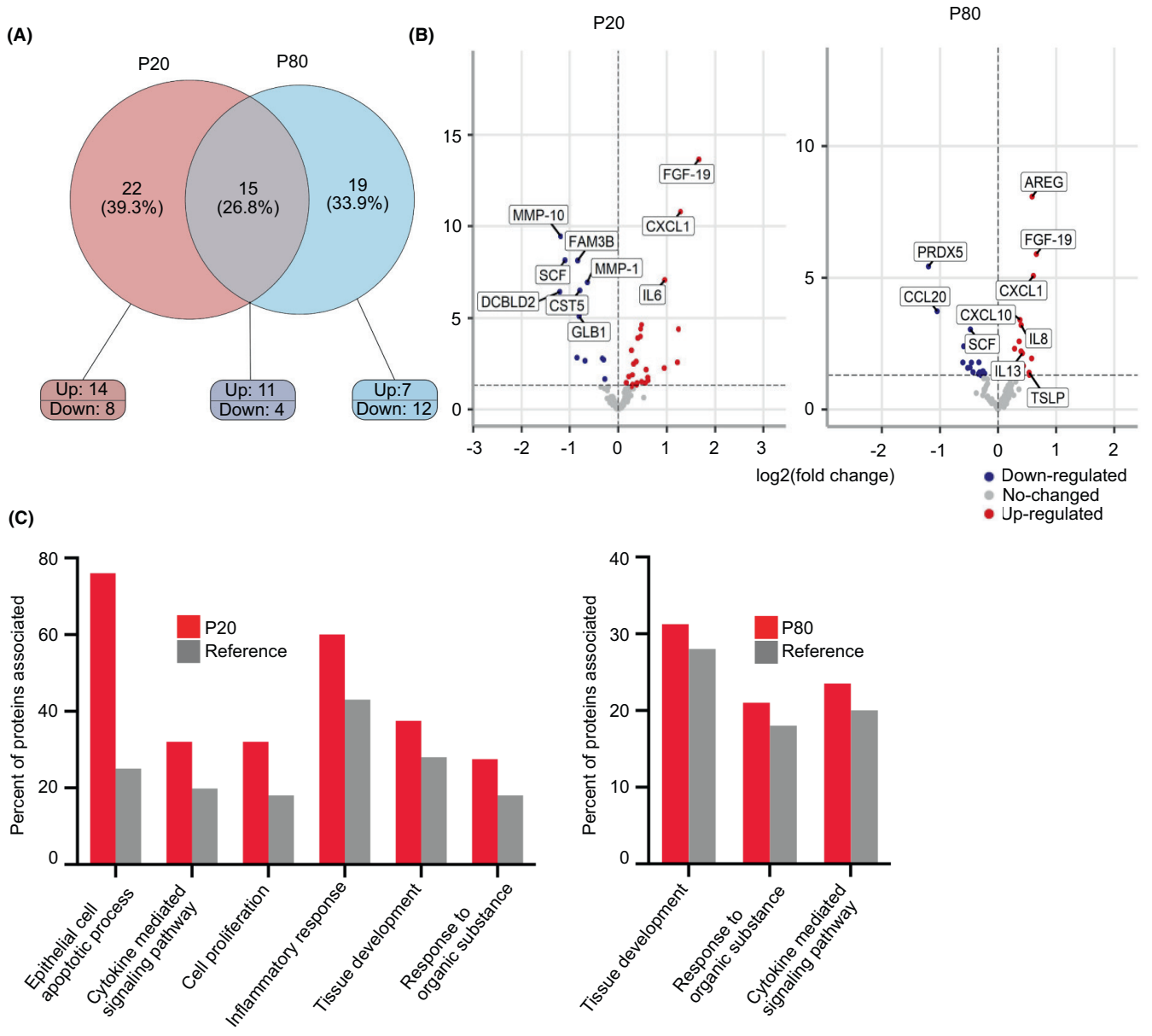


FIGURE 5 Summary of the targeted proteomics results in response to P20 and P80 in supernatants of monolayer-cultured Caco-2 cells. (A) Venn diagram shows the number of differentially expressed proteins in response to P20 ($n=5$) and P80 ($n=5$) at 0.05% concentration after 24 h. (B) Volcano plots illustrate differentially upregulated and downregulated proteins identified by using inflammation and immune response panels in response to P20 ($n=5$) and P80 ($n=5$). Upregulated genes are marked in red; downregulated genes are shown in blue. (C) Biological processes identified in inflammation and immune response panels in response to P20 and P80 at 0.05% concentration.

concentration induced dysbiosis in the small intestine, leading to enhanced vulnerability of the small intestine to indomethacin-induced injury. Their results suggested that P80-induced microbiota alteration might be one of the reasons why the incidence of inflammatory diseases of the small intestine is increasing worldwide. In addition, Jin et al.[24] reported that P80 (1%) intake could induce gut dysbiosis and promote susceptibility to colitis in adulthood. After these studies, there is a gap on the direct impact of food emulsifiers on epithelial barrier and expression at varying concentrations.

Several food emulsifiers and additives have been evaluated for their toxic effects, and many of them showed significant cytotoxicity in a concentration-dependent manner, such as P20,[30] P60,[30] P80,[31,32] carrageenan,[33] monosodium glutamate,[34] starch nanoparticles,[35] titanium dioxide,[36] allura red,[37] and tartrazine.[38] However, their molecular mechanisms by which these substances damage the epithelial barriers is not fully understood. Concentrations of P20 and P80 typically used in the food industry and daily exposure of humans demonstrated that these two non-ionic surfactants were cytotoxic at high doses, possibly by damaging the epithelial cell membranes. At concentrations between 1% and 0.1%, they both showed cytotoxic effects across the experimental models using iPSC-driven organoids, organo-chips and liquid–liquid interface cultures. The increases in the mRNA expressions associated with apoptosis and proliferation pathways have been demonstrated by both emulsifiers and P20

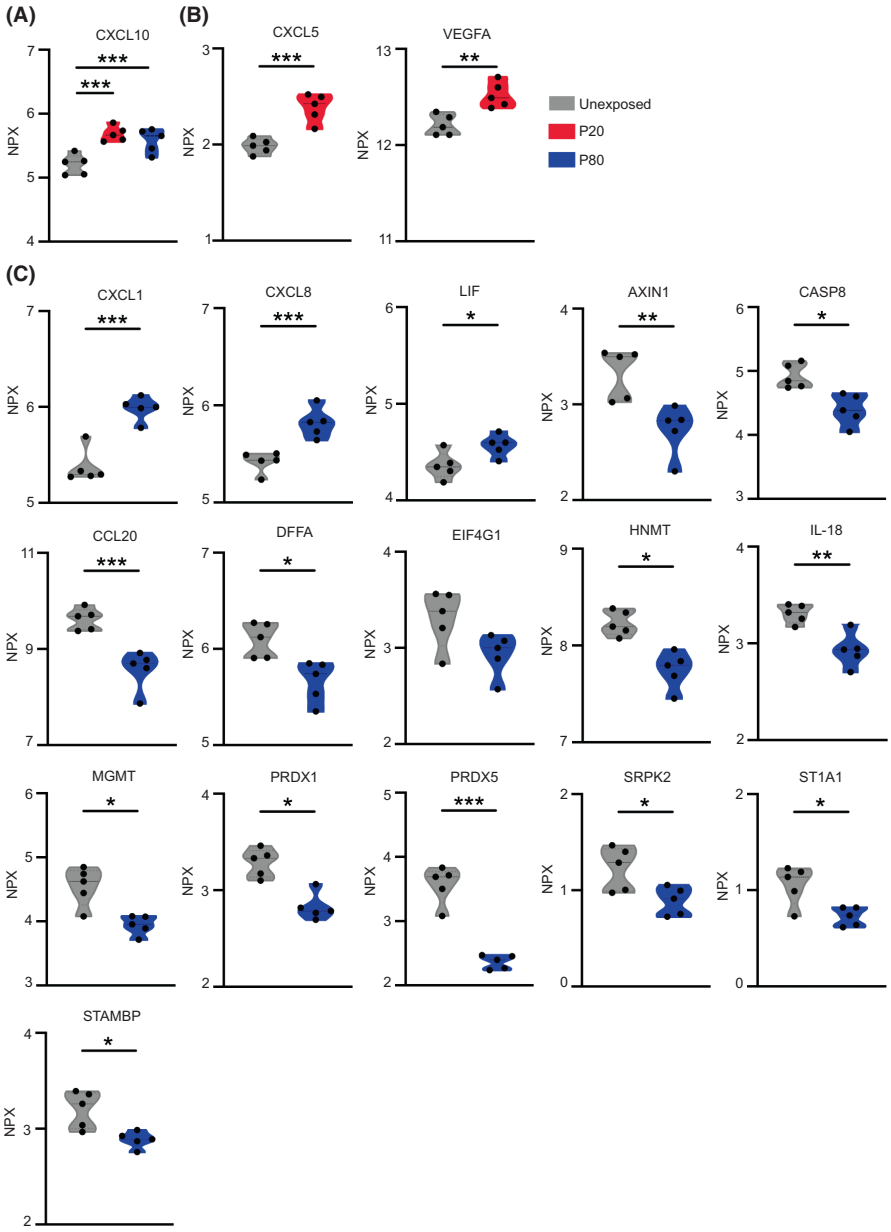


FIGURE 6 Differentially regulated hub proteins. Individual dots show the distribution of culture supernatant protein levels in response to P20 (A, B) and P80 (A, C) at 0.05% concentration. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$. NPX, normalized protein expression.

and P80 can upregulate apoptosis in the intestinal epithelium.[30,39] Our data further demonstrate that direct exposure of P20 and P80 alters mRNA expressions associated with developmental process, cell differentiation, cell communication, cell adhesion, cell migration, and response to stress. As indicated by our RNA-seq transcriptome data, the targeted proteome exhibited an upregulation in epithelial cell apoptotic process, cell proliferation, and inflammation response pathways upon exposure to P20. In addition, cytokine-mediated signaling pathway, tissue development, and response to organic substance pathways were observed in response to both P20 and P80.

The recently reported epithelial barrier theory establishes a link between several chronic inflammatory diseases and disrupted epithelial barriers. This has directed research interest to investigate the hazards of barrier-opening substances, such as food additives, in particular emulsifiers. Food emulsifiers can induce low-grade inflammation and have been demonstrated to contribute to obesity/metabolic syndrome, impair epithelial barriers, cause intestinal injury, alter the structure of the mucosal, modulate interactions between microbes and gastrointestinal tissues, instigate microbial dysbiosis, which may overall contribute to the development of intestinal inflammation.[19–23] Maternal intake of emulsifiers in mice was found to disrupt the intestinal barrier function, induces gut dysbiosis, and promotes susceptibility to colitis in adulthood.[24] Herein, we present strong evidence highlighting the critical roles of P20 and P80 in disrupting the intestinal barrier integrity in iPSC-derived human intestinal organoids, liquid–liquid interface cultures, and organ-on-a-chips. Although there was direct cellular toxicity at high doses, non-cytotoxic and relatively low doses were shown to have a direct impact on mRNA expressions of TJ and AJ proteins, increased proinflammatory chemokines and cytokines and their receptors, and activated PI3K-Akt and MAPK signaling pathways. In addition to the opening of epithelial barriers, one of the interesting effects of P20

and P80 was to initiate direct epithelial expression of proinflammatory genes, namely to cause epithelitis. Although not described in detail, the proinflammatory potential of emulsifiers and non-ionic surfactants has been previously reported. CMC and P80 were shown to significantly increase the expression of CXCL-1.[39] Carrageenan, an extensively used food additive, stimulates an inflammatory cascade in normal colonic epithelial cells via activation of B-cell lymphoma/leukemia 10 with nuclear factor kappa B (NF-κB) activation and upregulation of CXCL-8 (IL-8) secretion.[40] Recently, we reported that professional dishwasher rinse aid including non-ionic surfactant can activate NFκB as well as the AP-1 and MAPK pathways leading to increased proinflammatory activity in the gut epithelium.[41]

In pathway analysis from RNA-seq data, P20 influenced the ferroptosis and p38 MAPK signaling pathways, along with alanine, aspartame and glutamate metabolism and the Wnt signaling pathway, while P80 altered p38 MAPK signaling, PPAR-linked lipid metabolism, aryl hydrocarbon receptor (AHR) pathway, cellular senescence, extracellular matrix (ECM) proteoglycans and the arachidonate generation from diacylglycerol (DAG). These pathways can potentially affect various facets of epithelial cell functionality and the epithelial barrier's integrity. The AHR pathway's involvement suggests that P80 exposure could influence the regulation of genes related to barrier maintenance and defense responses, potentially affecting barrier function and responsiveness to environmental hazards.[42,43] Cellular senescence, potentially resulting from oxidative stress, may have an effect on barrier integrity maintenance. It is important to note here that inhibition of oxidative stress rescues epithelial barrier damage caused by detergents and surfactants.[44] The ECM proteoglycans pathway, which plays a role in cell adhesion, migration, and signaling, could affect the structural integrity of the epithelial barrier.[45] Alterations in this pathway due to P80 exposure may impact the barrier's capacity to maintain structural integrity and appropriate cellular interactions. Lastly, the arachidonate generation from DAG pathway, responsible for producing bioactive lipid mediators involved in inflammation, indicates that P80 might influence epithelial cell inflammatory responses and immune process regulation within the barrier.[46] In addition, proteome pathway analysis revealed that while P20 treatment elicits a Th1-prone immune response, P80 treatment initiates a Th2-driven response indicative of tissue restoration, cellular senescence due to oxidative stress and potentially decreased ROS scavenging proteins, aligning with prior research on P80's potential to increase oxidative stress susceptibility by reducing glutathione levels.[47]

Weighted co-expression network analysis was performed to visualize the overall picture of differentially expressed proteins by identifying the key modifying players (hub proteins) in response to P20 and P80. Since they have many interacting partners within a network, hub proteins have been considered as functionally significant.[48] As hub proteins, here, we highlight three upregulated cytokines and chemokines, CXCL5, CXCL10, and VEGF-A, associated with P20, and four upregulated proteins, CXCL1, CXCL8 (IL-8), CXCL10, and LIF, associated with P80 exposures. CXCL5 plays an important role in promoting angiogenesis, mediating inflammatory response and participating in connective tissue remodeling.[49] CXCL10 is regarded as a chemoattractant preferentially for activated T cells and particularly Th1 cells.[50] As inflammatory chemokines, CXCL5 and CXCL10 have been associated with inflammatory diseases such as Chron's disease and ulcerative colitis.[51] VEGF-A is an important regulator of angiogenesis and mediates most of the steps in the angiogenic cascade in endothelial cells, including proliferation, migration, and tube formation.[52] A recent study reported that CXCL5 promotes angiogenesis via activating the AKT/NF-κB/FOXD1/VEGF-A pathway in a CXCR2-dependent manner.[53] Since CXCL1 and CXCL8 are critical components of inflammation mediated processes, aberrant regulation of them and their receptors, CXCR1 and CXCR1/2 respectively, have been implicated in a number of inflammatory-mediated diseases.[54,55] CXCL1 and CXCL8 are also involved in modulating the proliferation, angiogenesis as well as migration of malignant cells.[56] LIF, a member of the IL-6 family, is a pleiotropic cytokine characterized by its paradoxically opposite effects in different cells.[57] In intestinal epithelial cells, LIF mainly activates STAT3, thus promoting proliferation. Studies have shown that the LIF expression level in the colon increases significantly in patients with ulcerative colitis.[58]

Several studies reported that commonly used food emulsifiers, such as polysorbate 80, carboxymethylcellulose (CMC), alter microbiota that promote intestinal inflammation.[17,19,59,60] They can disrupt the gut microbiota and these effects may facilitate the translocation of LPS from the gut lumen into the bloodstream, leading to low-grade inflammation and other health issues. After consuming emulsifiers, chronic intestinal inflammation together with intestinal bacterial encroachment was induced in germ-free mice.[59] More specifically, the administration of CMC or P80 to mice resulted in microbiota encroachment into the mucus; alterations in microbiota composition, including an increase of bacteria that produce proinflammatory flagellin and LPS and the development of chronic inflammation.[17,19,60,61] The levels of flagellin and LPS were enhanced after treatment with emulsifiers. Metagenomics results have demonstrated an enrichment of genes related to flagella and bacterial motility in the gut microbiome.[59,62] Research conducted on mice has also shown that the consumption of emulsifiers can alter the composition of the gut microbiota, increase intestinal inflammation, and promote metabolic disorders.[19] P80 has been associated with mucosal microstructure and particle dispersion.[21] It also increases the motility of *Escherichia coli* and its ability to translocate across microfold epithelial cells, through which the gut epithelium was invaded by intestinal floras.[13] However, it is worth noting that studies in humans are limited, and further research is needed to fully understand the impact of food emulsifiers on gut health and their potential connection to bacterial LPS.

Intact epithelial barriers are crucial for maintaining homeostasis as they protect host tissues from infections, environmental toxins, pollutants, and allergens. Under this light, we must consider that current toxicity levels established several decades ago may be outdated. It is imperative that we evolve our definition of “toxic” and identify safer alternatives for the barrier-damaging agents currently in use. The present study focuses on the molecular toxicity and the first phase of tissue damage induced by using low doses of emulsifiers

that cause "epithelitis." In conclusion, this study provides new insights into the underlying mechanisms of intestinal epithelial barrier defects in response to commonly used food emulsifiers P20 and P80. Our results demonstrate that P20 and P80 cause cell death at high doses and affect the maintenance of intestinal epithelial barrier function, expression, and regulation of TJ and AJ proteins and epithelial barrier damage, and induce a proinflammatory response even at low doses. At the cellular level, cell development, signaling, proliferation, apoptosis, inflammation, and response to stress were altered.

AUTHOR CONTRIBUTIONS

IO and CAA: study concept, methodology and design; IO, DY, YP, ENB, HB, SA, AH, and BR: acquisition of data; IO, DY, YP, ENB, HB, and SA: analysis and interpretation of data; KN and CAA: obtained funding; IO, RD, MA, KN, and CAA: study supervision; IO and CAA: drafting of the manuscript; DY, YP, ENB, HB, SA, AH, BR, VS, RD, MA, and KN: critical revision of the manuscript for important intellectual content.

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CONFLICT OF INTEREST STATEMENT

CAA is the Co-Chair for EAACI Guidelines on Environmental Science in Allergic diseases and Asthma and serves on the Advisory Boards of Sanofi/Regeneron, Novartis, Seed Health, GlaxoSmithKline, and SciBase, and is the Editor-in-Chief of Allergy. KN is Director of the World Allergy Organization Center of Excellence for Stanford, Advisor at Cour Pharma, Consultant for Excellergy, Red tree ventures, Eli Lilly, and Phylaxis, Co-founder of Before Brands, Alladapt, Latitude, and IgGenix; and National Scientific Committee member at Immune Tolerance Network (ITN), and National Institutes of Health (NIH) clinical research centers, outside the submitted work; patents include, "Mixed allergen composition and methods for using the same," "Granulocyte-based methods for detecting and monitoring immune system disorders," and "Methods and Assays for Detecting and Quantifying Pure Subpopulations of White Blood Cells in Immune System Disorders." RD is a co-founder and CEO in Seed, a biotechnology company. The rest of the authors declare that they have no relevant competing 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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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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