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Maternal periodontitis is associated with altered breast milk cytokines and reduced infant weight gain

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Abstract.

Maternal periodontitis is a prevalent inflammatory condition known to impact systemic health, yet its influence on breast milk composition and infant development remains underexplored. We conducted a prospective study of 36 mother-infant pairs, assessing maternal periodontal status, breast milk cytokine profiles, and infant weight gain at one month. Here we show that mothers with periodontitis exhibit elevated TNF-α levels in mature breast milk, which inversely correlate with early infant weight gain. In vitro, exposure of human intestinal epithelial cells to milk from periodontitis-affected mothers disrupted tight junction protein organization, reduced intestinal alkaline phosphatase activity, and increased epithelial permeability; these effects were partially reversed by TNF-α neutralization. These findings suggest that maternal periodontal inflammation may alter breast milk inflammatory signaling, potentially impairing neonatal intestinal barrier function and growth. This study supports a maternal oral-milk axis linking maternal oral health to postnatal intestinal homeostasis and early infant development.

1. Introduction

Periodontitis, a chronic inflammatory disease affecting more than half of adults worldwide, is a leading cause of irreversible destruction of the tissues supporting the teeth and represents a significant public health challenge [1]. Beyond its local clinical manifestations, the ulcerated periodontal pocket provides a persistent entry point for inflammatory cytokines and bacterial components, allowing them to enter the bloodstream and contribute to a sustained systemic inflammatory response [2].

Epidemiological studies have clearly linked periodontitis to adverse pregnancy outcomes, including preeclampsia, preterm birth, and low birth weight [3].

Two principal mechanisms have been proposed to explain this association during pregnancy: (i) the systemic spread of bacteria and their products to the placenta and fetal circulation, and (ii) the transfer of maternal inflammatory mediators (such as prostaglandin E2, IL-6, IL-1, and TNF-α) that can impact the fetal-placental unit [2]. Although the effects of maternal periodontitis on pregnancy outcomes are well documented, whether maternal periodontal inflammation can also affect infant health after birth through breastfeeding remains largely unexplored.

Adequate nutrition during the first 1,000 days of life, from conception to two years of age, is crucial for both short- and long-term health outcomes in offspring. Breast milk provides essential nutrients and a variety of bioactive factors, including immune cells, antibodies, cytokines, hormones, and microRNAs, which guide neonatal mucosal and immune maturation [4,5].

Maternal health conditions such as obesity, diabetes, and mastitis are known to alter breast milk composition and impact infant development [6-8]. However, the potential for chronic maternal oral

inflammation to alter milk inflammatory signaling and subsequently influence neonatal gut physiology, remains largely unexplored.

Early-life intestinal barrier integrity is critical for nutrient absorption, immune homeostasis, and physical defense. Disruption of this barrier is associated with infant growth failure, increased paracellular permeability, and systemic immune dysregulation [9].

Here, we investigated whether maternal periodontitis alters breast milk inflammatory profiles and whether these changes are associated with early infant growth and intestinal epithelial barrier function. Using a prospective cohort of mother-infant pairs, we quantified inflammatory mediators in maternal saliva, colostrum, and mature milk, and correlated cytokine levels with infant weight gain at one month. To explore underlying mechanisms, we exposed human intestinal epithelial (Caco-2) monolayers to mature breast milk and assessed effects on tight junction organization, intestinal alkaline phosphatase (IAP) activity, and paracellular permeability, including experiments with targeted TNF-α neutralization. Our findings provide proof-of-concept evidence for a maternal oral-milk axis, suggesting that maternal oral health may influence postnatal epithelial homeostasis and early infant growth through alterations in breast milk composition.

2. Material and methods

2.1 Study Design. This study was designed as a prospective observational pilot cohort study combined with mechanistic *in vitro* experiments to investigate whether maternal periodontal inflammation influences the inflammatory composition of breast milk and neonatal intestinal barrier function and growth. The human cohort included 36 mother-infant pairs recruited prospectively. The cohort comprised postpartum women classified according to periodontal status. For mechanistic studies, differentiated human intestinal epithelial (Caco-2) cells were exposed to individual breast milk samples. *In vitro* experiments used biological replicates from independent breast milk samples and technical replicates within each experiment.

The sample size was determined by feasibility and exploratory objectives, as this study was designed as a pilot proof-of-concept investigation. No formal power calculation was performed. Data collection was completed once the target recruitment period ended and all enrolled participants had completed follow-up. Outliers were not excluded from the analyses unless due to technical failure or incomplete data collection. No statistical outlier exclusion criteria were predefined, and all valid biological data points are reported. As this was an observational cohort study, participants were not randomized to exposure groups. *In vitro* experiments were conducted with investigators blinded to clinical group allocation during outcome assessment and data quantification whenever feasible.

2.2 Sex as a biological variable. This study included human mother-infant pairs in which all participating mothers were female by definition. Neonatal sex was recorded for all infants (male and female) and is reported in Table 1. Both male and female infants were included in analyses of growth outcomes, and no sex-based exclusion criteria were applied. Due to the limited sample size and the exploratory nature of this pilot cohort, the study was not powered to detect sex-specific differences in

infant outcomes; therefore, stratified analyses by infant sex were not performed. In the *in vitro* experiments, Caco-2 cells (a human epithelial cell line) were used to model intestinal barrier function.

2.3 Study approval. This prospective observational cohort study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethics Committee for Clinical Experimentation of the Tuscany Region – South East Vast Area Section (Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana – Sezione: AREA VASTA SUD EST), based at the Azienda Ospedaliero-Universitaria Senese / University of Siena (protocol code: Flo-Lat), submitted by the Principal Investigator, Prof. Filiberto M. Severi. Before collecting maternal and neonatal data and samples, all participating women provided written informed consent both for themselves and on behalf of their infants.

2.4 Study Population. The study was performed on saliva, colostrum and mature milk in mother-infant pairs followed up to 1 month postpartum. Women (n= 36) aged >18 years who had given birth live infants at term by planned cesarean section due to podalic presentation of the fetus or previous cesarean section or, by natural vaginal delivery, were recruited during the immediate postpartum period (within 24-48 hours after delivery) by the Division of Obstetrics and Gynecology, Department of Molecular and Developmental Medicine (University Hospital of Siena, Siena, Italy). Exclusion criteria included clinically diagnosed infections, taking antibiotics during the recruitment process, and fetal genetic defects. Periodontal status was assessed during the same postpartum hospitalization, within 24-48 hours after delivery, at the Division of Obstetrics and Gynecology by a calibrated examiner using the Periodontal Screening and Recording (PSR) system with a UNC periodontal probe. Study samples of saliva and colostrum were collected from mothers at 24-48 hours postpartum and the mature milk at a follow-up visit (1-month postpartum) at the Division of Obstetrics and Gynecology, Department of Molecular and Developmental Medicine (University Hospital of Siena, Siena, Italy). Data on maternal demographics and personal anamnesis were collected during the baseline enrollment

interview. Gestational age at delivery, infant sex, birth weight, and birth length were extracted directly from clinical medical records. At the 1-month postpartum follow-up visit, infant health status (hospitalizations and antibiotic use) and primary feeding type were recorded via maternal self-report. Exclusive breastfeeding was operationally defined according to World Health Organization (WHO) criteria. Feeding classification was established at the 1-month visit based on maternal recall covering the period from birth to follow-up. Infant anthropometric measurements at the 1-month follow-up visit were conducted directly by calibrated clinical staff, with infant body weight measured on a digital pediatric scale following a standardized protocol (infants weighed nude). All women enrolled underwent a periodontal screening record (PSR) performed at 24–48 hours postpartum by a calibrated examiner with a UNC periodontal probe. The probing used 6 sites per tooth, and a specific code value ranging from 0 to 4 was formulated for each sextant. The value of each sextant refers to the clinical condition: score 0, healthy periodontal conditions or no periodontal disease (black band completely visible or probing depth <3.5 mm); score 1, gingival bleeding (black band completely visible or probing depth <3.5 mm); score 2, calculus/defective margins and bleeding (black band completely visible or probing depth <3.5 mm); score 3, probing depth of 3.5-5.5 mm (black band partially visible) and a score 4, probing depth >5.5 mm (black band entirely within the pocket). Participants with PSR scores of 3 or 4 were subsequently evaluated by complete periodontal charting to confirm the diagnosis.

2.5 Samples collection and processing. Participant flow and availability of biological samples throughout the study are summarized in Figure 1. Saliva samples were collected at recruitment, 24-48 hours after delivery, using the Salivette collection device (Sarstedt Inc., Nuembrecht, Germany), before Periodontal Screening. The women were asked to gently chew the swab for 30-45 seconds and then put it back in the device's tube. The samples were centrifuged within 1 hour of collection and stored at −20° C until subsequent cytokine determination.

Samples of colostrum (1 ml; 24-48 hours after birth) and mature milk (15 ml; 1 month after birth) obtained in the morning by manual pumping or with a breast pump were collected in sterile polypropylene tubes and processed within one hour. Colostrum samples were obtained from 26 non-periodontitis and 10 periodontitis mothers. Mature milk samples collected at one month postpartum were available from 17 non-periodontitis and 7 periodontitis mothers, as the remaining participants had discontinued breastfeeding and transitioned to formula-feeding.

Five ml of mature milk was immediately frozen in polypropylene tubes at -80° C and used for subsequent *in vitro* gastrointestinal digestion. The remaining sample and colostrum were fractionated by centrifugation at 5,000 ×g for 15 minutes at 25° C to obtain three phases: cell pellet, a middle aqueous phase and the supernatant containing the lipids; the aqueous phase of both sample types (colostrum and mature milk) was used for the dosing of cytokines of interest and the mature milk sample was used for the *in vitro* experiments.

2.6 Cytokines quantification. Saliva, the aqueous phase of the colostrum, and mature milk were used to determine IL-1β, IL-6, MIF, and TNF-α by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). The limits of detection (LOD) specified by the manufacturer were 1.0 pg/mL for IL-1β, 0.70 pg/mL for IL-6, 16 pg/mL for MIF, and 1.6 pg/mL for TNF-α. All assays were performed in duplicate for each participant and according to the manufacturer's instructions.

2.7 *In Vitro* Gastrointestinal Digestion. Five ml of mature milk was digested *in vitro* by adding simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) according to the INFOGEST protocol [10].

Briefly, for the simulation of the salivary phase, a mix of mature milk and SSF (1:1; w/w), α-amylase (diluted 1:20), and $CaCl_2$ (1:200) were added and stirred for 2 min. Then the obtained bolus was added with SGF (1:1; w/w), pepsin (1:12.5), $CaCl_2$ (1:2000) and HCl 1M (to reach pH 3), then stirred for 1 h

163 at 37° C. The gastric chyme was added with SIF (1:1; w/w), pancreatin (1:80), bile salts (1:16), $CaCl_2$
164 (1:500) and NaOH 1 M (to reach pH 7), then the solution was stirred for 1 h at 37° C.

165 **2.8 Caco-2 cell culture and treatment.** The human colorectal adenocarcinoma cell line Caco-2
166 (Merck KGaA, Darmstadt, Germany) was grown in Dulbecco's modified Eagle's medium (DMEM)
167 supplemented with 20% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL
168 streptomycin (complete medium; all components are from Thermo Fisher Scientific Inc, Waltham,
169 MA, USA) in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C to a confluence of 50-60 %.
170 The cells used in the experiments were between passages 60 and 70. The Caco-2 cells were seeded in
171 6- or 96-well culture plates ($4.5x10^4$ and $2.5x10^3$ cells/well, respectively) or into polyester, 0.4-µm
172 pore size, 0.33 cm^2 culture surface, transwell Thincert (Greiner Bio-One, Milano, Italy)
173 ($3x10^3$cells/well). After 21 days of differentiation (complete medium changed every 2 days), cells were
174 washed twice in phosphate-buffered saline (PBS) and incubated for 72 hours in complete medium
175 containing 5% (v/v) aqueous milk phase or *in vitro*-digested aqueous milk phase at 37° C in a
176 humidified atmosphere of 95 % air and 5 % CO_2; controls were maintained in complete medium only.
177 At the end of incubation, depending on the experiment, cells were analyzed for viability using the
178 sulforhodamine B (SRB) assay as described by Orellana and Kasinski [11], for protein expression of tight
179 junction proteins (TJ) including occludin and zonula occludens-1 (ZO-1) by Western blot and
180 immunofluorescence, intestinal alkaline phosphatase (IAP) (protein expression and activity), and for
181 transepithelial barrier function using 70kDa-FITC-dextran permeability measurements.

182 **2.9 Intracellular ROS quantification**. Intracellular ROS content was assessed using 2',7'-
183 dichlorodihydrofluorescein diacetate acetyl ester (DCFH-DA; Invitrogen). Cells were differentiated
184 and then incubated with 5% of milk-digested or non-digested samples for 72 hours. At the end of the
185 treatments, cells were rinsed twice with PBS and incubated with 10 µM DCFH-DA probe for 30 min
186 at 37°C. Cells were harvested by scraping, and dichlorofluorescein (DCF) fluorescence intensity was

measured at 480/528 nm using the Synergy HTX multi-mode reader (BioTek, Winooski, VT, USA). DCF fluorescence was normalized to the viability obtained by the SRB assay.

2.10 Protein Extraction and Western Blot Analysis. Caco-2 cells were lysed in Mammalian Protein Extraction Reagent (MPER)® buffer (Thermo Fisher Scientific Inc.) with the addition of 1% (v/v) of Halt™ Protease and Phosphatase Inhibitor Single-Use cocktail (Thermo Fisher Scientific Inc.). Protein content was determined using the Bradford assay, and 40 µg of total protein was electrophoresed at a constant voltage of 135 V for 75 min in TGS buffer (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS) on an 8% or 10% acrylamide/bis-acrylamide gel. The separated proteins were then electro-transferred to nitrocellulose membranes, followed by a 1 hour incubation at room temperature in blocking buffer (20 mM Tris, pH 7.5; 500 mM NaCl, 5% nonfat dry milk, 0.1% Tween-20) and subsequent incubation with blocking buffer containing primary antibodies rabbit anti-human IAP (Abcam, Cambridge, UK), rabbit anti-human ZO-1 and rabbit anti-human occludin antibodies, used 1:1000 (Cell Signaling, Danvers, MA, USA); mouse anti-human actin used 1:2000 (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4° C. The membranes were then washed and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated IgG (BioRad, Hercules, CA, USA). The reaction was detected with a chemiluminescence reagent (BioRad), and the bands were digitized with CHEMIDOC Quantity One 1D (BioRad). Densitometric analyses were completed using ImageJ software.

2.11 Immunofluorescence. In other experiments, cells were seeded on rounded slides in 6-well plates ($4.5x10^4$ cells/well) and, after full differentiation, treated for 72 hours at 37° C with complete medium with or without 5% aqueous milk phase. After incubation, slides were washed twice in PBS, fixed in paraformaldehyde 4% for 10 minutes, then permeabilized with TritonX100 at 0.2% for 5 minutes; blocked with 1% bovine serum albumin (BSA) for 45 minutes, and finally incubated overnight with rabbit anti-human occludin antibody (Cell Signaling) 1:100 in PBS, rabbit anti-human ZO-1 antibody

(Cell Signaling) 1:100 or rabbit anti-human IAP (1:100) (Abcam) in PBS. Slides were then washed with PBS and incubated for 1 hour with goat anti-rabbit IgG Alexa Fluor 488 (Thermo Fisher Scientific Inc.), diluted 1:1000 in PBS. Nuclei were counterstained with propidium iodide as described by Suzuki *et al*. [12] with minor modification. Images were acquired on a Zeiss LSM700 confocal microscope.

2.12 Intestinal alkaline phosphatase activity. Intestinal alkaline phosphatase (IAP) activity was assessed by measuring the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol (pNP), which produces a yellow color detectable at 405 nm, as described by Ferruzza *et al.* [13]. The assay was performed on Caco-2 cell lysates.

In brief, treated and untreated Caco-2 cells were washed twice with 1 mL of PBS and then scraped into 300 µL of cold non-denaturing lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Triton X-100). Lysates were sonicated in three 10-second cycles with cooling intervals on ice between pulses to prevent thermal denaturation, and then centrifuged at 13,000 × g for 15 minutes at 4 °C. The resulting supernatants were collected as crude extracts for intracellular IAP quantification. For the enzymatic assay, 20 µL of cell lysates was incubated with 180 µL of pNPP substrate solution (2.5 g/L pNPP in 100 mM diethanolamine buffer, 150 mM NaCl, 2 mM $MgCl_2$, pH 9.5). After 8 minutes at room temperature, the reaction was stopped by adding 50 µL of 0.5 M NaOH. Absorbance was measured at 405 nm using a Multiskan FC plate reader (Thermo Fisher Scientific Inc.). Background correction was applied by subtracting appropriate blanks consisting of samples incubated with substrate solution lacking pNPP. IAP activity was normalized to the protein concentration of the cell extracts, and results were expressed as fold change relative to untreated Caco-2 cell cultures.

2.13 Transepithelial barrier function. Caco-2 cells were plated on permeable transwell membranes (0.4-µm pore size), and after 21 days of culture, the cells were treated with 5% (v/v) aqueous milk phase or digested aqueous milk phase from healthy and periodontitis-affected mothers. In parallel

experiments, anti-human TNF-α antibodies (150 μg/mL) (R&D Systems) were added to the aqueous milk phase of samples from mothers with periodontitis. At the end of exposure (72 hours), FITC-Dextran 70 (70kDa; Merck KGaA) (1 mg/mL) was added to the top of the monolayers, and after 210 minutes, the fluorescence flux in the basolateral side media and the standard curve of FITC-Dextran 70 (1-0.2 μg/mL) from which the results were extrapolated were measured, using the fluorescence reader (Synergy HTX multi-mode reader, BioTek, Winooski, VT, USA). The permeability index was calculated as described by Sato *et al*. [14].

2.14 Statistical analysis. All datasets were assessed for normality using the Shapiro-Wilk test. Comparisons between two independent groups were performed using the unpaired Student's t-test or Mann–Whitney U test, as appropriate. For comparisons involving more than two groups, one-way ANOVA followed by Tukey's multiple-comparison test or the Kruskal-Wallis test was used, as appropriate. Longitudinal changes in cytokine concentrations between paired colostrum and mature milk samples were assessed using the Wilcoxon matched-pairs signed-rank test in participants with paired samples (n = 17 non-periodontitis and n = 7 periodontitis). Spearman correlation analysis was used to assess associations between milk cytokine concentrations and infant weight gain at one month. Exploratory multivariable linear regression analysis was performed using the ordinary least squares method, with infant weight gain at one month as the dependent variable and milk TNF-α concentration, maternal BMI, and smoking status as independent variables. Regression coefficients (β), 95% confidence intervals, and corresponding p values were estimated. Model assumptions were evaluated by assessing residual normality and multicollinearity using variance inflation factors.

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The level of significance was set at $p \leq 0.05$.

3 Results

3.1 Maternal periodontitis is associated with elevated TNF-α levels in breast milk and reduced infant weight gain

A total of 36 mother–infant pairs were enrolled, with mothers delivering either by planned cesarean section or vaginal delivery, including 26 mothers without periodontitis and 10 mothers with periodontitis. Colostrum and saliva samples were obtained from all participants. At one-month postpartum, mature milk samples were collected from all mothers who were exclusively breastfeeding (n = 17 non-periodontitis and n = 7 periodontitis). The remaining participants (n = 9 non-periodontitis and n = 3 periodontitis) had transitioned to formula-feeding (Figure 1).

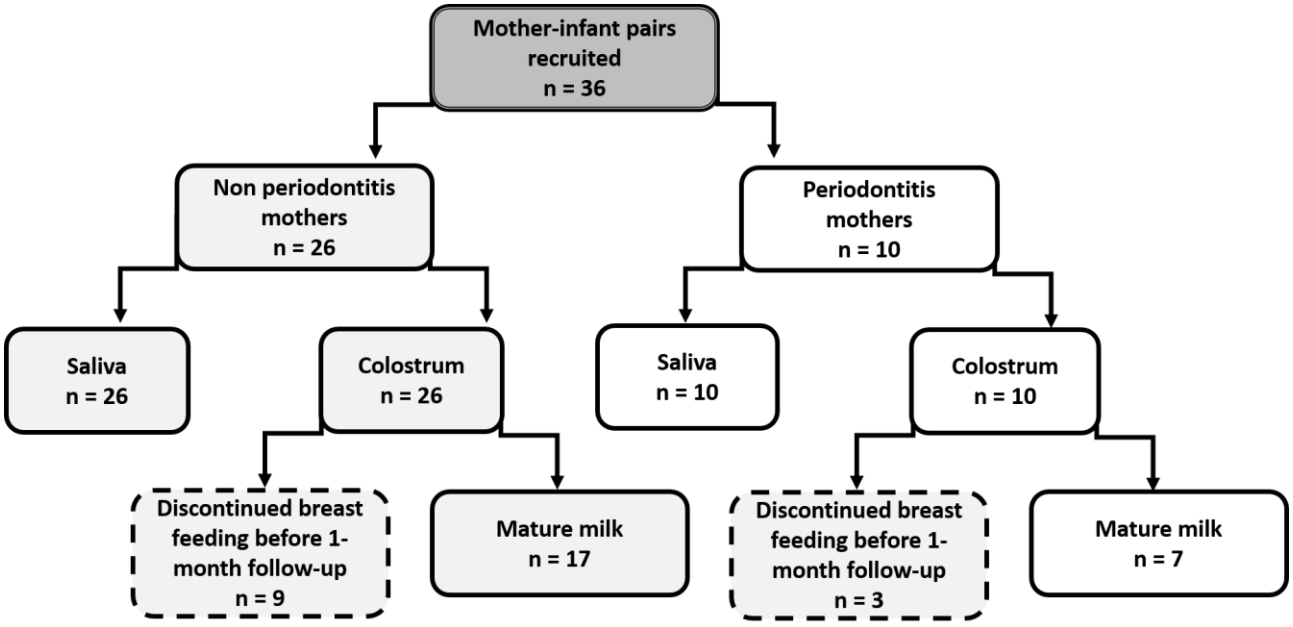


Figure 1. Participant flow diagram. Flow of mother–infant pairs through the study. Thirty-six mother-infant pairs were enrolled, including 26 mothers without periodontitis and 10 mothers with periodontitis. Colostrum and saliva samples were collected from all participants at baseline. At one-month postpartum, mature milk samples were available from 17 mothers without periodontitis and 7

mothers with periodontitis. The remaining participants had discontinued breastfeeding and transitioned to formula feeding before follow-up sample collection.

Maternal and neonatal characteristics are summarized in Table 1. No significant differences were observed between healthy and periodontitis-affected mothers regarding body mass index (BMI) or gestational age at delivery. Two of ten mothers with periodontitis developed gestational diabetes mellitus. Neonatal birth weight and overall weight gain at 1 month did not differ between groups when analyzed without stratification by feeding type. Exclusive breastfeeding was reported in 66% of participants, while 34% of women reported inadequate breast milk production and moved to formula-feeding. When infants were stratified by maternal periodontal status, those exclusively breastfed by mothers with periodontitis showed significantly lower weight gain at one month compared with infants of healthy mothers ($p < 0.05$) (Table 1).

283 **Table 1. Maternal and infant Characteristics.** BMI: Body Mass Index; GDM: gestational diabetes

284 mellitus.

Maternal and infant Characteristics n= 36 women	Healthy (n=26)	Periodontitis (n=10)	p
Maternal age (years ± SD)	35± 3	35± 1	ns
Pre-pregnancy weight (kg ± SD)	63.5 ± 9.2	66.7± 9.2	ns
Maternal BMI (±SD)	23.6 ± 4.0	24.8 ± 2.6	ns
Maternal weight increment (kg ± SD)	12.6 ± 2.0	11.0 ± 3.0	ns
Pregnancy complication		GDM n=2	
Mode of delivery: **natural delivery** **caesarean section**	 19 7	 6 4	
Gestational age at delivery (weeks ± SD)	38.9± 2	39 ± 1	ns
Infant sex	Male=12 Female=14	Male=6 Female=4	ns
Infant birth weight (g ± SD)	3249 ± 308	2975 ± 505	ns
Infant weight increment at one month (g ± SD)	869 ± 440	630 ± 311	ns
Feeding: **breast-feeding** **formula-feeding**	 n=17 n=9	 n=7 n=3	
Infant weight increment at one month of breast-feeding (g ± SD)	1041 ± 424 (n=17)	655 ± 360 (n=7)	p=0.047
Infant weight increment at one month of formula-feeding (g ± SD)	545 ± 252 (n=9)	573 ± 196 (n=3)	ns

285

No significant differences were observed among formula-fed infants. Periodontal clinical variables are reported in Table 2.

Table 2. Overall oral health-status of periodontitis mothers. FMPS: full mouth plaque score; FMBS: full mouth bleeding score; PPD: probing pocket depth; DMFT: decayed missing and filled teeth.

Periodontal clinical variables	Observations (n = 10)
FMPS (Mean ± SD)	79.5 ± 11.23
FMBS (Mean ±SD)	45 ± 13.28
PPD 1-3 mm (Mean ± SD)	111.5 ± 14.27
PPD 4-5 mm (Mean ± SD)	53.2 ± 11.85
PPD ≥ 6 mm (Mean ± SD)	9.3 ± 3.77
DMFT (Mean ± SD)	11.4 ± 6.40
Smokers (N [%])	4 (40)
N of cigarettes per day (Mean ± SD)	8.75 ± 4.79
Familiarity to Periodontitis (N [%])	4 (40)
Taste alterations (N [%])	2 (20)
Periodontitis Stage and Grade (N [%])	
3A	4 (40)
3B	2 (20)
3C	2 (20)
4B	1 (10)
4C	1 (10)

Mothers with periodontitis had elevated full-mouth plaque scores (FMPS) and full-mouth bleeding scores (FMBS) based on Papapanou *et al*. [15], consistent with advanced disease stages. Results indicate that approximately 28% of women had periodontitis with PSR score of 3 or 4 (PSR ≥ 3), including two participants with a PSR score of 4 and eight with a PSR score of 3. Forty per cent of these mothers were smokers.

Participants not classified as having periodontitis had PSR scores ranging from 0 to 2, consistent with periodontal health or mild pregnancy-associated gingival inflammation.

Analysis of saliva samples revealed significantly higher levels of the proinflammatory cytokines IL-1β and MIF in mothers with periodontitis compared to healthy controls ($p < 0.0001$ and $p < 0.001$, respectively). IL-1β levels were increased approximately tenfold, and MIF levels were nearly doubled in the periodontitis group as shown in Figure 2. Both cytokines exhibited a broader distribution and greater inter-individual variability among mothers with periodontitis, consistent with the heterogeneous inflammatory burden associated with periodontal disease. TNF-α and IL-6 were not included in the analysis because their concentrations in saliva samples were below the reliable detection range of the assay.

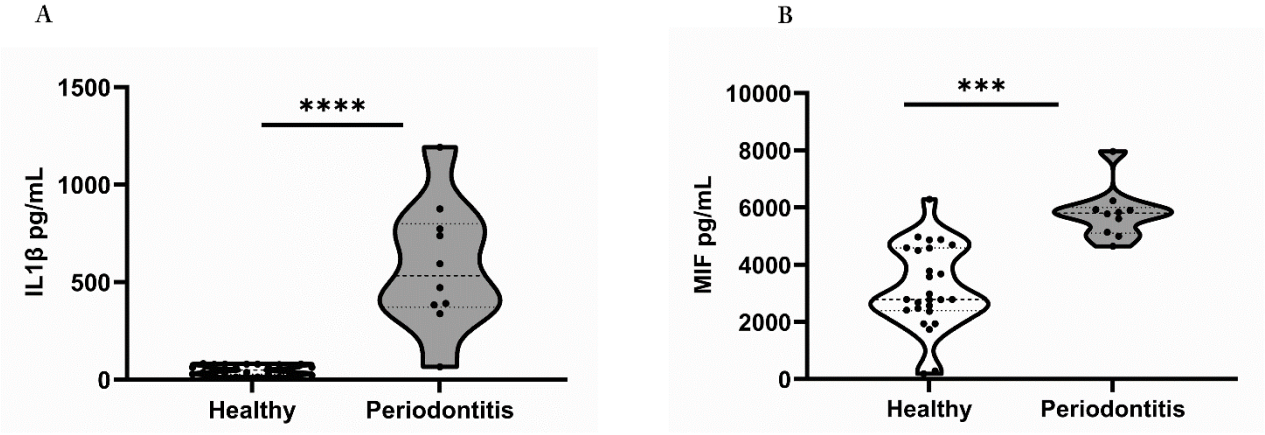


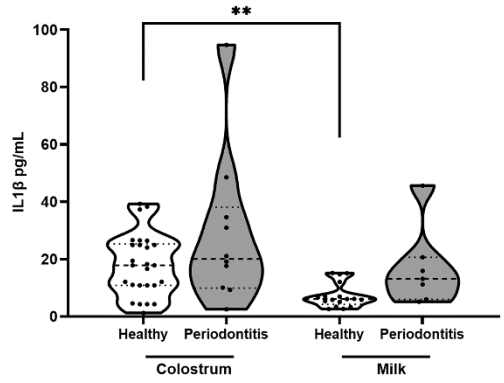
Figure 2. Cytokine profiles in saliva of healthy and periodontitis-affected mothers. A) IL-1β and B) MIF cytokine levels (pg/ml) in saliva samples of healthy (n=26) and periodontitis (n=10) postpartum women. Data are presented as violin plots showing individual data points, with dashed lines representing the median and dotted lines representing the interquartile range (25^{th} and 75^{th} percentiles). Statistical significance was determined using the Mann–Whitney U test. ***$p < 0.001$; ****$p < 0.0001$.

We then examined the concentrations of IL-1β, MIF, IL-6 and TNF-α (Figure 3A-D) in colostrum and mature milk collected one month after birth. In non-periodontitis mothers, paired analysis showed a significant decrease in IL-1β, MIF, and TNF-α concentrations from colostrum to mature milk, whereas IL-6 concentrations did not show a statistically significant longitudinal change. No significant longitudinal changes were observed in mothers with periodontitis. Importantly, TNF-α concentration in mature milk was significantly higher in mothers with periodontitis (n = 7) than in healthy mothers (n = 17) ($p < 0.05$; Figure 3D). In addition, TNF-α levels in the periodontitis group displayed greater

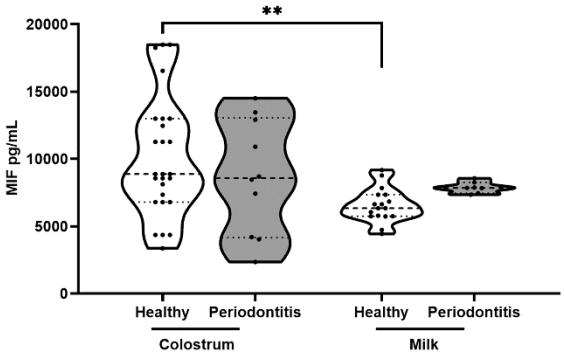
inter-individual variability and a distribution shifted toward higher values compared with healthy controls.

Correlation analysis showed a significant inverse relationship between TNF-α levels in mature milk and infant weight gain at one month (Spearman $r = -0.42$; $p = 0.042$) (Figure 3E), indicating that higher TNF-α concentrations were associated with reduced postnatal growth. In an exploratory multivariable linear regression model adjusted for maternal BMI and smoking status, the inverse association between milk TNF-α concentration and infant weight gain remained statistically significant ($\beta = -157.4$; 95% CI: −292.8 to −22.1; $p = 0.025$), whereas maternal BMI and smoking status were not significant predictors. Importantly, no adverse clinical events were reported in any infants at the follow-up visit, aside from the observed differences in weight gain.

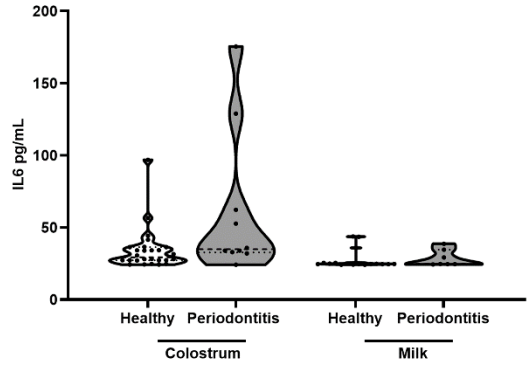
A


**
IL1β pg/mL
100
80
60
40
20
0
Healthy
Periodontitis
Healthy
Periodontitis
Colostrum
Milk

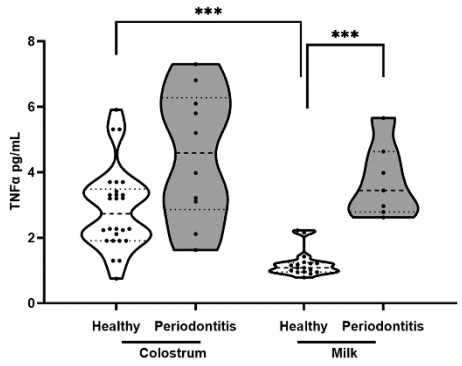
B


**
MIF pg/mL
20000
15000
10000
5000
0
Healthy
Periodontitis
Healthy
Periodontitis
Colostrum
Milk

C


IL6 pg/mL
200
150
100
50
0
Healthy
Periodontitis
Healthy
Periodontitis
Colostrum
Milk

D


***
***
TNFα pg/mL
8
6
4
2
0
Healthy
Periodontitis
Healthy
Periodontitis
Colostrum
Milk

E

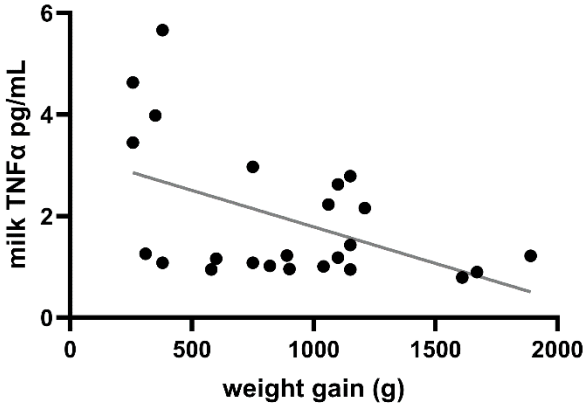
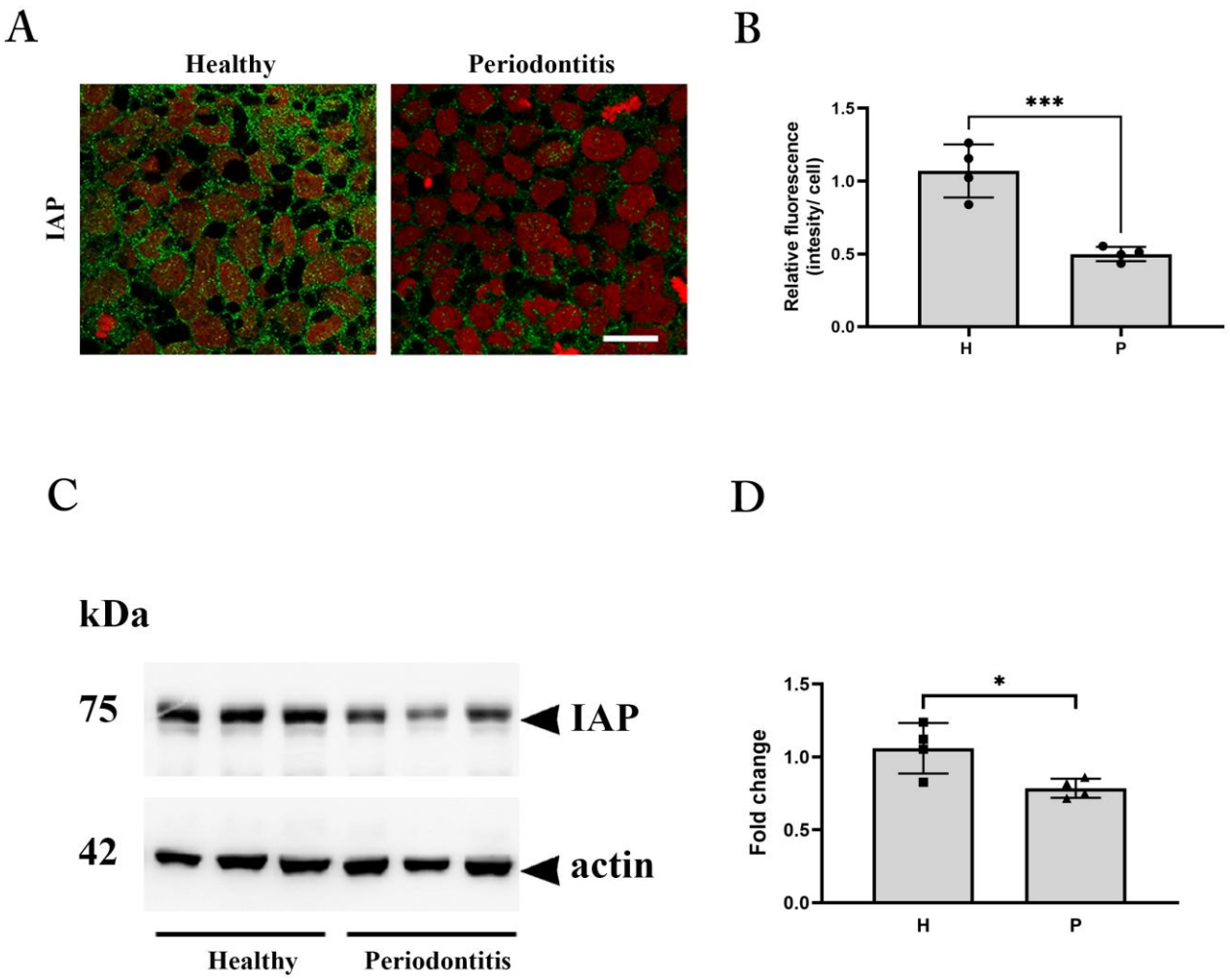

milk TNFα pg/mL
6
4
2
0
0
500
1000
1500
2000
weight gain (g)

Figure 3. Cytokine profiles in colostrum and mature milk of healthy and periodontitis-affected mothers. Concentrations of IL-1β (A), MIF (B), IL-6 (C), and TNF-α (D) were quantified by ELISA in colostrum and mature milk from non-periodontitis and periodontitis-affected mothers. Data are presented as violin plots showing individual data points, with dashed lines representing the median and dotted lines representing the interquartile range (25^{th} and 75^{th} percentiles). Longitudinal comparisons between colostrum and mature milk in participants with paired samples (n = 17 non-periodontitis and n = 7 periodontitis) were performed using the Wilcoxon matched-pairs signed-rank test, whereas between-group comparisons were performed using the Mann–Whitney U test. **p<0.01; ***p<0.001. (E) TNF-α concentrations in mature milk (n = 17 non-periodontitis and n = 7 periodontitis) were inversely associated with infant weight gain at one month (y = – 0.001438x + 3.225; Spearman r = – 0.42; p = 0.042).

3.2 Periodontitis-associated breast milk alters intestinal barrier protein expression *in vitro*

To test whether breast milk from mothers with periodontitis impairs intestinal barrier function, differentiated Caco-2 monolayers were exposed to aqueous milk samples from healthy or periodontitis-affected donors. After 72 hours of exposure, cell cultures were evaluated for the expression of IAP and the TJ proteins ZO-1 and occludin by confocal microscopy and Western blot analysis.

Cells treated with milk from healthy mothers showed robust IAP expression localized along the apical membrane, whereas those exposed to milk from mothers with periodontitis exhibited markedly reduced IAP staining (Figure 4 A-B). This reduction was confirmed by Western blot and densitometric analysis, which demonstrated a statistically significant downregulation of IAP protein levels in the periodontitis group compared to the healthy group (Figure 4 C-D), supporting the immunofluorescence findings.



358

359

360 **Figure 4. Reduced IAP expression in intestinal epithelial cells exposed to periodontitis-associated**
361 **breast milk.** (A) Representative immunofluorescence of IAP (green) in Caco-2 cells cultured for 72
362 hours with mature milk samples from healthy or periodontitis-affected mothers. Nuclei are
363 counterstained with propidium iodide (red); Bar=20 μm; (B) Quantification of IAP fluorescence
364 intensity per cell performed using healthy (H; n = 4, independent donor samples) and periodontitis (P;
365 n = 4, independent donor samples) -associated milk samples; (C) Representative Western blot and (D)
366 corresponding densitometric analysis of IAP protein levels in Caco-2 cells exposed to healthy (H; n =

4, independent donor samples) and periodontitis (P; n = 4, independent donor samples) -associated milk samples. Significance was determined with an unpaired t-test for two-group comparisons. Data are shown as mean ± SD. *$p < 0.05$; ***$p < 0.001$.

TJ organization was also impaired. ZO-1 and occludin displayed continuous apical distribution in cells exposed to healthy milk but became fragmented and discontinuous at the cell edges following treatment with periodontitis milk (Figure 5 A). Quantitative analysis of immunofluorescence and Western blots demonstrated significant downregulation of both TJ proteins (Figure 5 B and D) in Caco-2 cells exposed to milk samples from periodontitis-affected mothers compared to healthy controls.

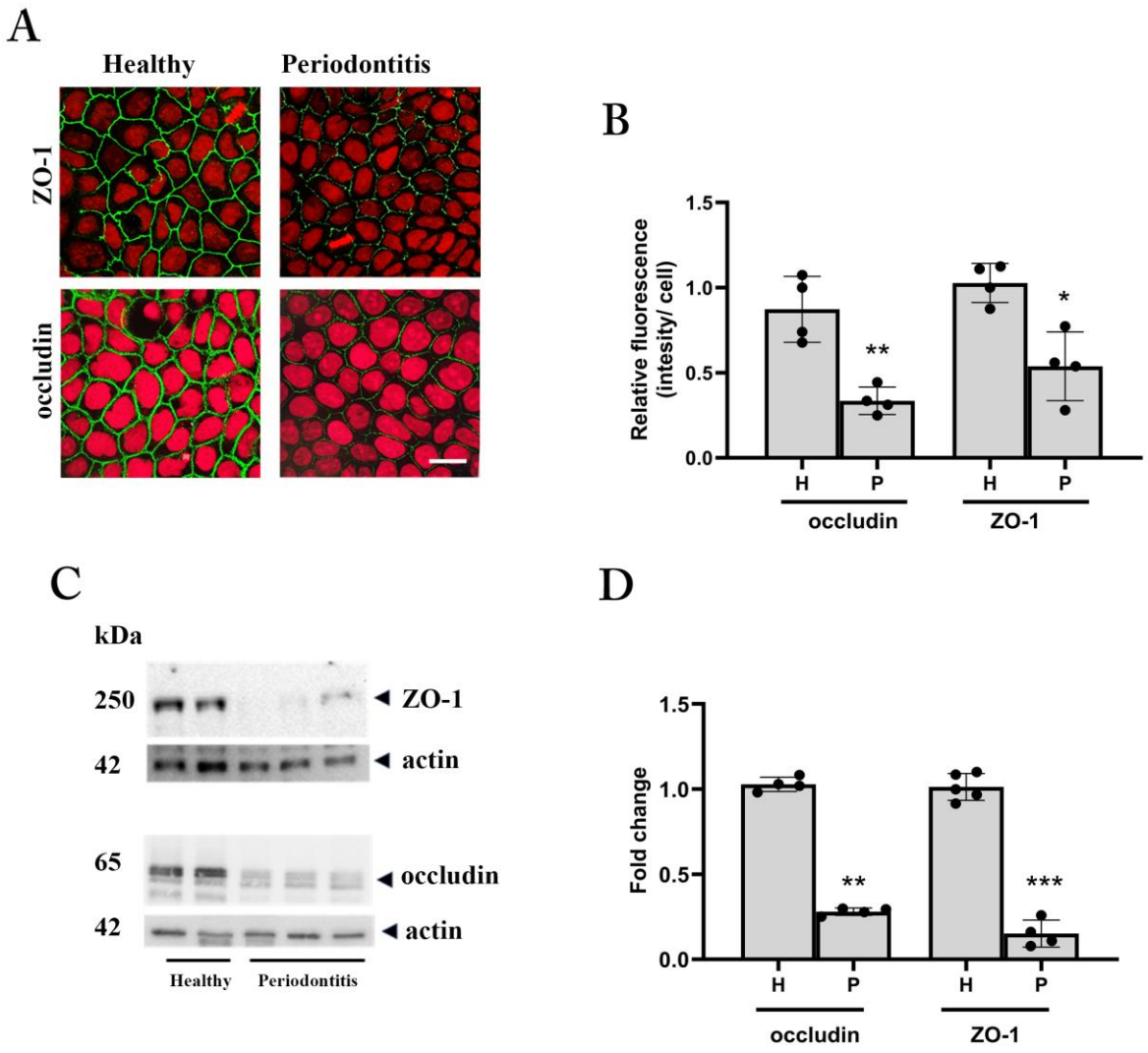


Figure 5. Disruption of tight junction protein expression in intestinal epithelial cells exposed to periodontitis-associated breast milk. (A) Representative immunofluorescence of ZO-1 and occludin (green) in Caco-2 cells cultured for 72 hours with mature milk from healthy or periodontitis-affected mothers. Nuclei are counterstained with propidium iodide (red). Bar= 20 µm. (B) Quantification of ZO-1 and occludin fluorescence intensity per cell performed using healthy (H; n = 4, independent donor samples) and periodontitis (P; n = 4, independent donor samples) -associated milk samples; (C) Representative Western blot and corresponding densitometric analysis (D) of ZO-1 and occludin in Caco-2 cells exposed to healthy (H; n = 4, independent donor samples) and periodontitis (P; n = 4,

independent donor samples) milk. Significance was determined with an unpaired t-test for two-group comparisons. Data are shown as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

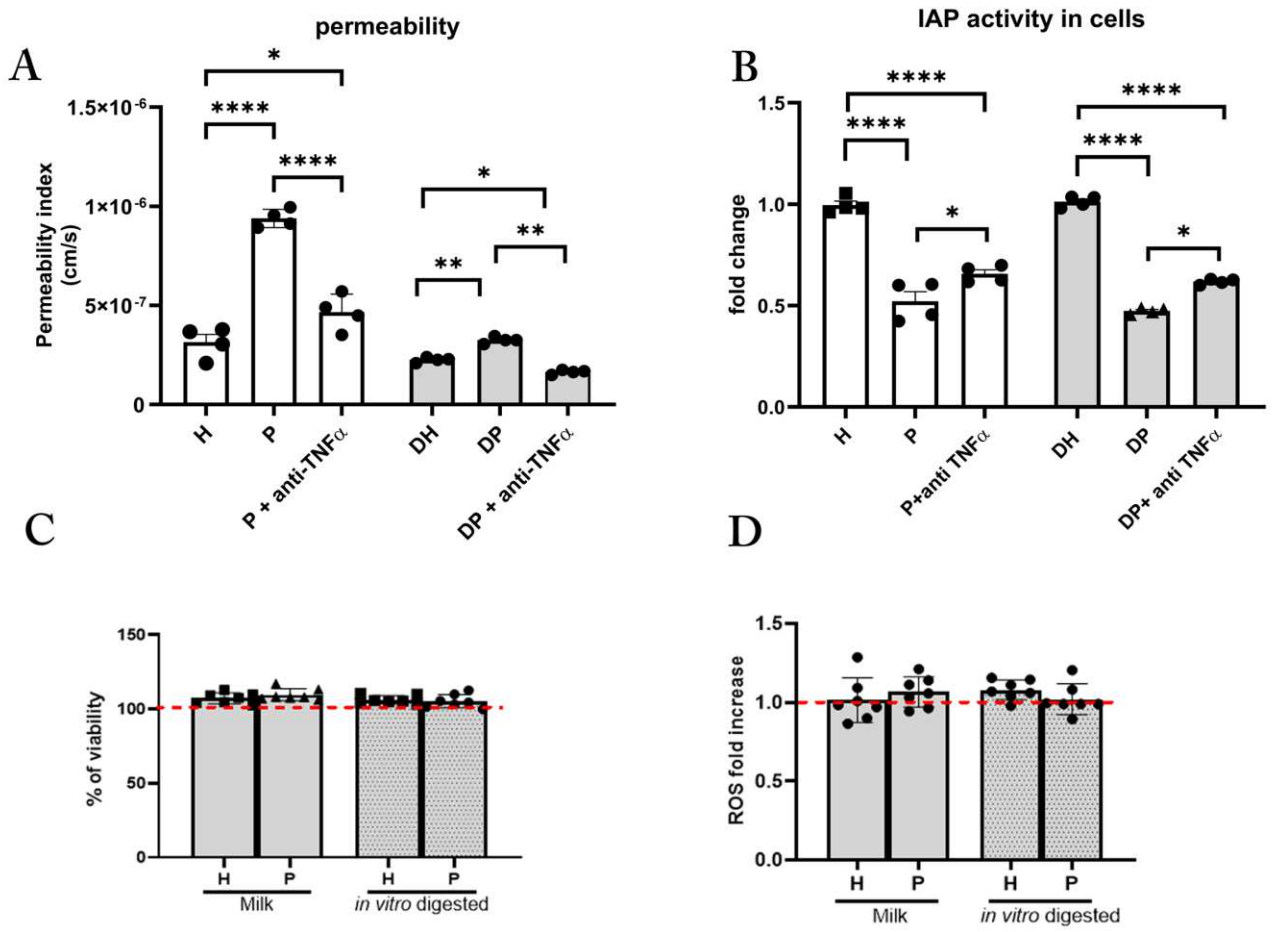
3.3 Periodontitis-associated breast milk alters epithelial permeability and reduces IAP activity *in vitro*

In functional experiments, the cells were differentiated in transwells and healthy or periodontitis milk was applied to the apical side for 72 hours.

Functional permeability assays revealed a corresponding loss of barrier integrity. The permeability index, resulting from FITC-dextran flux across Caco-2 monolayers, increased significantly after exposure to periodontitis milk compared to healthy controls (Figure 6 A, $p < 0.05$). Neutralization of TNF-α in periodontitis milk partially restored barrier integrity, reducing but not fully normalizing, permeability values ($p < 0.0001$ *vs* untreated periodontitis milk; $p < 0.05$ *vs* healthy milk).

Similar effects were observed with *in vitro* digested milk samples, which better mimic the *in vivo* situation, although the magnitude of disruption was lower (Figure 6 A).

Consistent with these findings, IAP enzymatic activity was significantly reduced in cells exposed to periodontitis milk relative to healthy milk, both before and after *in vitro* digestion (Figure 6 B, $p < 0.05$). Addition of anti- TNF-α antibodies partially reversed this suppression, confirming TNF-α as a key mediator of the inhibitory effect. Cell viability and ROS production remained unchanged across all conditions (Figure 6 C and D), indicating that barrier dysfunction was not due to cytotoxicity or oxidative stress.



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407

408 **Figure 6. Effect of milk from healthy and periodontitis mothers on intestinal barrier function**
409 **and cell viability.** (A) Permeability index calculated by measuring the 70-kDa FITC-dextran flux
410 across differentiated Caco-2 monolayers exposed to milk from healthy (H, n=4), periodontitis-affected
411 (P, n=4, independent donor samples) mothers or digested healthy milk (DH, n=4, independent donor
412 samples) and digested periodontitis milk (DP, n=4, independent donor samples). (B) IAP activity in
413 Caco-2 cells exposed to native or *in vitro* digested milk (DH, digested healthy; DP, digested
414 periodontitis) for 72 hours, expressed as fold change relative to healthy controls. (C) Cell viability and
415 (D) ROS levels in Caco-2 cells exposed to native (H, healthy, n=7 and P, periodontitis, n=7,
416 independent donor samples; representing all available periodontitis mature milk samples and an equal

number of matched healthy controls) or digested milk (H, healthy, n=7 and P, periodontitis, n=7) for 72 hours, expressed as percent or fold change relative to untreated controls (red line). Data represent mean ± SD from n = 4 or n = 7 independent biological milk samples per group, each assayed in technical triplicate. *p < 0.05; **p < 0.01; ****p < 0.0001 by one-way ANOVA with Tukey's post-test.

Collectively, these findings show that milk from mothers with periodontitis alters IAP expression and activity and disrupts tight junction protein organization *in vitro*, with TNF-α contributing to these effects. Together with the observed association between milk TNF-α levels and infant weight gain, these findings support a potential link between maternal oral inflammation, breast milk inflammatory signaling, and intestinal epithelial homeostasis.

4 Discussion

This study identifies an association between maternal periodontal inflammation and altered breast milk inflammatory composition during lactation. Breast milk from mothers with active periodontitis showed sustained elevation of TNF-α, which inversely correlated with infant weight gain during the first month of life. In *vitro,* exposure of intestinal epithelial monolayers to periodontitis-associated milk was associated with changes in tight junction organization, IAP expression and activity, and paracellular permeability. Targeted TNF-α neutralization partially rescued these epithelial changes.

Maternal health plays a critical role in fetal development, and this programming effect may extend beyond birth through breastfeeding. Although conditions such as obesity, diabetes, and mastitis are known to alter breast milk composition [16], the influence of maternal oral inflammation on milk immune signaling has not been defined. Our data suggest that periodontal inflammation may be associated with persistent immune activation during lactation, reflected by increased TNF-α in mature milk.

Paired longitudinal analyses demonstrated a significant decline in milk inflammatory mediators including IL-1, MIF and TNF-α during lactation among mothers without periodontitis, consistent with the expected transition from colostrum to mature milk. This pattern was not observed among mothers with periodontitis, suggesting that maternal periodontal inflammation may contribute to sustained immune activation during lactation. However, given the limited number of paired samples in the periodontitis group, these findings should be interpreted cautiously and warrant confirmation in larger cohorts.

A central clinical observation in this study was reduced weight gain among infants exclusively breastfed by mothers with periodontitis compared with those breastfed by healthy mothers. This difference occurred despite similar maternal BMI and gestational age, suggesting a possible role for

maternal inflammatory status. The absence of similar effects in formula-fed infants is consistent with a possible role for breast milk-derived inflammatory mediators.

Given that maternal smoking and BMI are well-known determinants of breast milk composition and infant growth [17], these variables were included as covariates in the exploratory analysis. The inverse association between milk TNF-α concentrations and infant weight gain persisted after adjustment for maternal BMI and smoking status. Nevertheless, due to the multifactorial nature of periodontal disease, residual confounding by unmeasured factors such as socioeconomic status, education, and other maternal characteristics cannot be excluded.

Newborns typically lose weight during the first days after birth and subsequently begin to regain weight [18]. In our cohort, infants of non-periodontitis mothers followed this expected pattern, whereas those of mothers with periodontitis gained approximately 20% of their birth weight by 30 days, compared with approximately 30% in the non-periodontitis group. This difference was more pronounced among breastfed infants, raising the possibility that differences in breast milk composition may contribute to the observed association. However, maternal periodontal inflammation could also influence infant outcomes through prenatal exposure to maternal oral bacteria or inflammatory mediators. Because fetal exposure was not directly assessed, our study cannot distinguish between prenatal and postnatal pathways, and the association between maternal periodontitis, milk TNF-α, and infant weight gain cannot be attributed specifically to breast milk. Nevertheless, the greater difference in weight gain among exclusively breastfed infants, together with the higher TNF-α concentrations in mature milk and the effects of periodontitis-associated milk on intestinal epithelial cells *in vitro*, is consistent with a potential postnatal contribution of breast milk. Prenatal and other maternal pathways may, however, act in parallel.

The prevalence of maternal periodontitis in our cohort (~28%) aligns with prior reports in the pregnant population [19,20], underscoring its clinical relevance. Saliva from affected mothers showed significantly elevated levels of IL-1β and MIF, both markers of periodontal activity and bacterial accumulation [21,22]. These findings confirm that local oral inflammation was active during lactation and may contribute to systemic immune activation.

Elevated TNF-α concentrations in breast milk provide a plausible postnatal mechanism through which maternal periodontal inflammation may influence intestinal epithelial function, given the established role of this cytokine in modulating epithelial barrier function [23,24]. To explore this possibility, we conducted *in vitro* studies using differentiated Caco-2 intestinal epithelial cells to assess the functional consequences of exposure to breast milk from mothers with and without periodontitis.

Previous *in vitro* studies with human intestinal organoids have shown that breast milk promotes epithelial differentiation, increases expression of TJ proteins such as occludin, and reduces permeability [25]. Disruption of these regulatory processes can compromise barrier integrity, leading to malabsorption and chronic inflammation [26]. Exposure of differentiated Caco-2 monolayers to milk from mothers with periodontitis resulted in marked disruption of epithelial organization and increased paracellular permeability, without changes in cell viability or oxidative stress, indicating that barrier dysfunction was mediated by cytokine signaling rather than cytotoxicity. TNF-α is a well-established regulator of epithelial permeability and tight-junction integrity, in intestinal epithelial models, TNF-α downregulates and redistributes ZO-1 and occludin, thereby increasing paracellular flux [27,28]. Animal studies further support this mechanism: TNF-α administration in mice induces redistribution of occludin and ZO-1 [29], while TNF-α deficiency attenuates the loss of these junctional proteins during experimental colitis [23]. Notably, milk from mothers with periodontitis also markedly suppressed the expression and enzymatic activity of IAP, a critical brush-border enzyme that detoxifies bacterial lipopolysaccharides (LPS), regulates luminal pH, and protects tight junction architecture [30]. Its

suppression, together with tight junction disassembly, suggests a potential multi-target compromise of epithelial defense.

Interestingly, neutralization of TNF-α in milk from mothers with periodontitis partially restored barrier integrity and IAP activity, supporting its role as a key contributor to the observed epithelial dysfunction. Because cell viability and oxidative stress levels were unaffected, these findings suggest that milk from mothers with periodontitis alters gut barrier function through specific cytokine-mediated signaling pathways rather than nonspecific cytotoxicity. As intestinal barrier function is closely linked to nutrient uptake and metabolic balance [31], its disruption may underlie the reduced postnatal growth observed in this study. In an *in vivo* context, such compromise of epithelial barrier integrity and reduced IAP activity could contribute to subclinical mucosal inflammation, altered nutrient absorption, and impaired metabolic efficiency, potentially explaining the diminished early growth seen in breastfed infants of mothers with periodontitis.

Several limitations warrant consideration. As an exploratory translational study, the modest clinical cohort size limited the number of covariates included in multivariable regression models to avoid overfitting. Although the inverse association between milk TNF-α levels and infant growth remained robust after adjustment, the observational design precludes definitive causal inference. Education, socioeconomic status, and other potentially relevant determinants of periodontal inflammation and infant growth were not comprehensively assessed, so residual confounding cannot be excluded. While Caco-2 monolayers effectively model epithelial transport and tight junction regulation, they do not fully recapitulate the complex architecture of the neonatal gut, including immune and microbial interactions. The partial, rather than complete, rescue observed following TNF-α neutralization suggests that other milk-derived inflammatory mediators or microRNAs may act synergistically. Finally, because prenatal exposure to maternal systemic inflammation was not directly quantified, we

cannot exclude parallel contributions to lower early weight gain resulting from intrauterine inflammatory exposure rather than solely from postnatal milk-derived factors.

In summary, our findings provide initial evidence for a potential postnatal link between maternal periodontal inflammation, alterations in breast milk composition, and early infant outcomes. Future prospective cohort studies and interventional trials investigating whether treatment of maternal periodontitis during pregnancy or lactation can restore normal breast milk composition will be important to confirm the clinical significance of the maternal oral-milk axis for neonatal health.

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Data and materials availability: The raw data supporting the conclusions of this article will be made available upon request to the corresponding authors, without undue reservation.

Author contributions:

Designed research: FMS, FI, MT

Methodology: SP, MT, GT, CB, GC

Conducted research: MV, LE, SN, RR, LC, ML, FB

Analyzed data or performed statistical analysis: SP, MT, MV

Interpreted results of experiments: FI, MT, SP, ND

Project administration: FMS, FI

Supervision: FMS, FI, ND

Writing-original draft: FI, MT, SP, ND

Writing-review & editing: FMS, FI, ND, LE

All authors read and approved the final manuscript

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Conflict of Interest Statement: Authors declare that they have no competing interests.

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